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THE PHYLOGENY AND DISTRIBUTION OF MITOCHONDRIAL DNA IN THREESPINE STICKLEBACK (*GASTEROSTEUS ACULEATUS*) ON THE QUEEN CHARLOTTE ISLANDS: EVIDENCE FOR PLEISTOCENE GLACIAL REFUGIA.

by

PATRICK O'REILLY

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

IN

ZOOLOGY

EDMONTON, ALBERTA

SPRING 1991

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Abstract

Mitochondrial DNA variation was analyzed in a total of 147 threespine stickleback (*Gasterosteus aculeatus*) from nine freshwater populations within the Queen Charlotte Islands (QCI), a population from the adjacent British Columbia mainland, two marine locations from the nearby Pacific Ocean, and a single site near Nova Scotia, from the east coast of Canada. All 11 haplotypes identified in specimens from the west coast of Canada grouped into two divergent assemblages (the pacific and rouge lineages), which were separated by a minimum of 0.0209 substitutions per base pair. The distinction between these two lineages is considerably greater than that observed between marine stickleback from the Pacific and those surveyed from Nova Scotia (0.0115 substitutions per base pair), 16,000 kilometres distant and within a separate ocean basin.

Pacific lineage haplotypes were observed in all marine fish surveyed off the QCI, as well as freshwater individuals from the adjacent mainland, Moresby Island, and most of Graham Island. The highly divergent rouge lineage was confined to four populations from the extreme northeast corner of Graham Island.

The distribution of haplotypes and levels of divergence observed between freshwater and marine stickleback suggests a recent marine origin (within the Holocene) of most lacustrine populations surveyed from marine stickleback, the putative ancestor of freshwater populations along the Pacific Coast of North America. Fish from the four northeast Graham Island localities surveyed, however, may have last shared a female common ancestor with marine stickleback prior to the Wisconsin. This suggests the persistence of freshwater populations within (or nearby) the northeast corner of Graham Island in ice-free refugia during a period when, according to geologists, the entire QCI was heavily glaciated.

Among the QCI freshwater populations surveyed were several distinct morphotypes, including a gigantic form greater than twice the length of typical freshwater stickleback, and a second type exhibiting marked lateral plate, spine, and pelvic girdle reduction or loss. The phylogeography of mtDNA haplotypes is consistent with a recent (post-glacial), polyphyletic origin of gigantism observed in several lakes, but suggests a possible monophyletic origin of the second morphotype, observed in four populations from two adjacent watersheds.

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I. Introduction

In many respects the flora and fauna of the Queen Charlotte Islands (QCI) are quite distinct from the biota on the adjacent British Columbia mainland. Endemism has been documented at the subspecies level in birds and mammals (see McCabe and Cowan 1945; Foster 1965), and to the species level in bryophytes (Schofield 1969), angiosperms (Calder and Taylor 1968), crustaceans (Bousfield 1958), and insects (Kavanaugh 1980). Also, marked regional morphological differentiation exists amongst QCI populations of deer mice (*Peromyscus maniculatus*, Rhoads 1894; Foster 1965), and threespine stickleback (*Gasterosteus aculeatus*); in fact, the range of variation reported in local populations of the latter is comparable to that observed throughout this species' entire circumboreal distribution (Moodie and Reimchen 1973, 1976b). Rapid post-glacial evolution has been postulated to account for the unique forms of some of these species (Foster 1965; Moodie and Reimchen 1976a), while others are suspected of having persisted in biotic refugia within the archipelago (Schofield 1969; Kavanaugh 1980; Cowan 1989; Lindsey 1989).

Another peculiar characteristic of the QCI biota, the disjunct distribution of some of the archipelago's botanical species, has been cited as evidence for refugia in the region (Lindsey 1989; Taylor 1989). Several flowering plants are found only on the QCI and northwest Vancouver Island (Ogilvie 1989), parts of which are also suspected of having escaped glaciation (Heusser 1960). Many bryophytes occur within the QCI and western Europe or southeast Asia, but not elsewhere within the western hemisphere (Schofield 1969, 1989). This kind of distribution is thought to be a result of extensive glaciation and the ensuing eradication from most of what was, in pre-Pleistocene times, probably a much more broad and continuous range. The pockets of persisting habitation likely represent areas which remained ice free.

In a geological survey of the QCI, Sutherland-Brown and Nasmith (1962) concluded that with the possible exception of high elevation nunataks, the entire archipelago was overlain by about 1,000 metres (m) of locally generated ice during latter stages of the Wisconsin, and suggested that perhaps biologists ought to seek some explanation other than persistence in refugia to account for the biotic anomalies observed in the islands. This conclusion was based on the following: 1) the freshness of erosional features; 2) the unweathered nature of glacial deposits; and 3) a single radiocarbon date of approximately 11,000 years B.P. from a peat sample at a depth of 6.6 m from Langura Island (see Heusser 1960).

More recent radiocarbon analyses and plant macrofossil data, however, indicate the sudden presence of aquatic and terrestrial floral communities at a site near Cape Ball (figure 1), approximately 16,000 years B.P., during and subsequent to the glacial maximum on the Pacific Coast of Canada (Warner et al. 1982). As the nearby mainland probably was covered by continental ice sheets at this time, source communities likely persisted somewhere within the QCI for the site to have been so rapidly colonized. From comparisons of historical sea levels on the QCI and nearby B.C. mainland coasts, Warner and colleagues also demonstrated that isostatic depression in the vicinity of Graham Island was considerably less than on the adjacent mainland, an indication of a thin localized ice cover in the area, as opposed to the thick and extensive ice sheets envisioned by earlier geologists.

Primarily owing to its maternal mode of inheritance and lack of recombination, mitochondrial DNA (mtDNA) is a sensitive marker of founder events (Wilson et al. 1985), and has been used to estimate Pleistocene or post-Pleistocene routes and times of colonization (Thomas and Beckenbach 1986; MacNeil and Strobeck 1987; Ashley and Wills 1987; Billington and Hebert 1988). Upon separation and subsequent isolation of formerly panmictic populations, random drift and natural selection will cause both nuclear and mitochondrial genomes to diverge. If the populations remain separate, the initial split will be recorded in

both nuclear and mitochondrial genomes. However, in the event of secondary contact, gene flow between populations may occur and eventually recombination will diminish the distinctiveness of the formerly divergent nuclear genomes. On the other hand, mtDNA markers of the isolation event will persist until one of the two distinct assemblages of haplotypes is lost via lineage sorting.

In a preliminary study, Gach and Reimchen (1989) surveyed mtDNA variation among four populations of threespine stickleback from the QCI, to examine patterns and times of colonization. They suggested that had freshwater populations persisted in isolation from each other previous to the maximum advance of the Wisconsin, a given level of interpopulation divergence in mtDNA sequences would be expected. Considerably fewer base substitution differences were observed between the two morphologically divergent lake populations surveyed than the number they calculated ought to have accrued had they separated during the late Wisconsin. Their results were consistent with radiocarbon dates of one of the two lakes and another in close proximity to the second lake surveyed (10,000-12,000 years B.P., Warner 1984).

Limited divergence between freshwater stickleback populations, however, does not necessarily imply that they are of recent origin (i.e., that they evolved from marine stickleback following deglaciation). Gene flow between two long isolated refugial populations and the extinction of one of the two divergent lineages, or post-Pleistocene colonization by a common refugial ancestor also could account for the genetic similarity. Prolonged habitation of the islands under various patterns of colonization would be detected more readily by focusing instead on comparisons between freshwater and marine stickleback.

Here, mtDNA variation is surveyed amongst freshwater populations of threespine stickleback *(G. a. leiurus)* from the Queen Charlotte Islands and adjacent mainland, and morphotypically marine stickleback *(G. a. trachurus)*, captured in estuarine or brackish waters

from the nearby Pacific and Atlantic oceans (figures 1 & 2). One objective of this report was to provide an independent evaluation of the refugium hypothesis originally proposed by biologists in response to the islands' disjunct floral distributions and high levels of morphological endemicity. Since comparisons between freshwater and marine stickleback were an important focus, mitochondrial genomes of 20 morphotypically marine stickleback from opposite ends of the study area (the west coast of Graham and east coast of Moresby islands) were characterized, as were the genomes of three individuals from the Atlantic west coast near Nova Scotia. Stickleback from a freshwater site on the British Columbia mainland (which was mantled by part of the Cordilleran glacier complex during the latter stages of the Wisconsin, Heusser 1960) were analyzed and compared with marine stickleback to contrast the magnitude of divergence observed between QCI *leiurus* and *trachurus* populations. The survey of QCI freshwater locations was expanded in this study over Gach's and Reimchen's earlier work, including extensive sampling of the northeast corner of Graham Island, within the area defined by Sutherland-Brown as the Argonaut Plain. Effort was concentrated in this location for the following reasons: 1) the proximity to pre-Holocene vegetative communities at Cape Ball; 2) the occurrence of several endemic populations which depart markedly from other marine or freshwater forms in the region (Reimchen 1984); 3) the presence of a population of stickleback exhibiting a unique symbiotic association with a previously undescribed dinoflagellate not found elsewhere (Reimchen and Buckland-Nicks 1990).

If extant lacustrine populations of threespine stickleback from the QCI were colonized following the Wisconsin glaciation, limited divergence between freshwater and marine stickleback would be expected. Greater levels of divergence, however, would indicate a more lengthy period of isolation from marine populations, presumably within glacial refugia in or nearby the QCI archipelago.

Analysis of mtDNA variation also has provided insight into the phylogenetic relationships and morphological evolution of several groups of closely related organisms. For example, Lansman et al. (1983) found that individuals of the two long recognized morphotypes of deer mice, the short tailed, short eared variety common to grasslands and the long tailed, long eared type generally associated with forested environments (Blair 1950), do not belong to two separate evolutionary clades, at least with respect to their maternal lineages. Instead, patterns of mtDNA variation reflected the broad scale geography of North America. This supports suggestions that differences in tail and ear length are adaptations to the different selection pressures imposed by forest and grassland environments (see Avise et al. 1987a).

In a survey of mtDNA from 37 species of cichlid fish from several of the African rift valley lakes Meyer et al. (1990) present strong evidence for a monophyletic origin of intra-lacustrine species flocks (including Lake Victoria assemblages); these findings indicate that similar morphotypes and similar specializations observed in the different lakes evolved independently on several occasions. This is in contrast to a polyphyletic origin arrived at from analyses of morphological characteristics (see Greenwood 1980); under this scenario, extant assemblages were presumed to have descended from groups of cichlids which were morphologically differentiated prior to the formation of the rift valley lakes (see Avise 1990).

The second objective of this study was to investigate the origin and evolution of several QCI stickleback populations that depart considerably from marine or common freshwater morphotypes observed in the region. Generally, *trachurus* have laterally compressed, elongate bodies, tapering anteriorly to a small mouth, and posteriorly to a slender, keeled caudal peduncle. They lack scales, but possess a complete series of bony lateral plates (30-35 per side, Hagen 1967). *Trachurus* is also characterized by three dorsal spines, a pair of prominent ventral spines, and a single anal spine. *Leiurus* are typically similar to the marine

form described, but can be distinguished by the absence of a keel, a deeper body, and smaller overall size (as indicated by standard length measurements of adult males and females, see Hagen 1967). In addition, they generally have fewer lateral plates; the number varies between four and 35, but in most populations is usually between five to ten (Reimchen 1983).

In Drizzle and Mayer lakes, on north central and south central Graham Island, respectively (figure 2), dark melanistic individuals nearly twice the length of typical freshwater stickleback were found (Moodie and Reimchen 1973; Moodie and Reimchen 1976b). Giant stickleback, though much lighter in body colour, also occur in Skidegate Lake, (north Moresby Island, Moodie and Reimchen 1976b). The majority of fish surveyed from Boulton Lake (north central Graham Island) by Moodie and Reimchen (1973) were virtually lacking pelvic girdles and pelvic spines, and showed loss or major reduction in dorsal spines. Stickleback from the northeast Argonaut Plain lakes surveyed (Harelda, Imber, Rouge, and Serendipity) lack lateral plates, exhibit major spine reduction, and have a reduced pelvic girdle; in fact, individuals from Serendipity lack any vestiges of a pelvic girdle whatsoever (Moodie and Reimchen 1976b; Reimchen 1984; T. Reimchen, personal communication). In the most morphologically divergent population, Rouge, in addition to extreme pelvic girdle and ventral spine reduction, fish lack an anal spine, have a postcranial hump, and some are without middle dorsal fin rays, resulting in two dorsal fins (Reimchen 1984). Stickleback from Van and Shrimp lakes, located on the west coast of Graham Island and the west coast of the adjacent mainland, respectively (figure 1), resemble the typical Pacific Coast freshwater stickleback described by Hagen and Gilbertson, except individuals of the latter population are more fully plated and slightly smaller in overall body size (T. Reimchen, personal communications).

Miller and Hubbs (1969) suggested that morphological variation amongst threespine stickleback populations may be a product of the species' survival in different glacial refugia. If the endemic QCI populations surveyed here were indeed descended from groups of

stickleback which were morphologically differentiated prior to deglaciation of the region, then phenotypic similarities observed in several populations (for example, the gigantism observed in Mayer and Drizzle, or plate loss and spine reduction documented in the Argonaut Plain localities) may be due to common ancestry, as opposed to parallel or convergent evolution implicated by McPhail and Lindsey (1970) and Bell (1976) in proposing that stickleback in different watersheds along the west coast of North America were derived independently from marine stickleback.

On the basis of geography and morphological variation, Reimchen et al. (1985) concluded that Drizzle and Mayer populations did evolve independently from marine stickleback, and inferred that gigantism observed in the two localities is an example of convergent evolution. However, as selection may rapidly mould the *Gasterosteus* phenotype (see Moodie and Reimchen 1976a; Reimchen 1980, 1983; Reimchen et al. 1985), morphology may not be a reliable indicator of phylogenetic relationships or patterns of colonization; this would also appear to be so for African cichlid fish as demonstrated by Meyer et al. (1990). Assessments of mtDNA variation amongst stickleback from relevant populations is useful here because, unlike many morphological differences, most mtDNA mutations are not highly visible to selection (Clark and Lyckegaard 1988; see also Avise et al. 1987a), and therefore patterns of mtDNA variation should reflect more accurately phylogenetic relationships amongst QCI stickleback populations and historical patterns of colonization.

II. Materials and Methods

A. Study area

The Queen Charlotte archipelago is a triangular group of islands approximately 80 kilometres (km) from the adjacent British Columbia mainland, and about 50 km from the nearest costal islands. The two islands surveyed here (Graham and Moresby, figure 2), are the largest and most northerly of the group. Excluding Van Lake, all of the sites surveyed from Graham Island are well within the Queen Charlotte Lowlands, an area extending from the most southern waters of Masset Inlet to the northeast corner of Moresby Island (Sutherland-Brown 1968). Generally less than 150 m in elevation, the lowlands typically are covered by muskeg, yellow pine and small spruce. Most of the lakes within the region are shallow, and because they are surrounded by sphagnum bog, are characteristically highly stained and have a low pH. Boulton Lake, however, is quite clear as the lake has no inflow streams but rather is maintained via ground water seepage (Gach and Reimchen 1989). Northeast of a line between Masset Harbour and Cape Ball, is a subdivision of the lowlands called the Argonaut Plain (Sutherland-Brown 1968; see figure 2). The western border of this area represents the furthest easterly progress of late Wisconsin glaciers originating from the Queen Charlotte range of mountains to the west (Sutherland-Brown 1968). The Argonaut Plain is a late Pleistocene outwash plain deposited by run off from the melting glaciers. Rouge, Serendipity, Imber, Harelda, and Drizzle lakes are within the boundaries of the plain as defined above. However, the presence of glacial erratics near Drizzle Lake (T. Reimchen, personal communication) suggests that the border may have been drawn a little too far to the east in this area; therefore, this lake will be excluded in all future references to the Argonaut Plain. Van Lake is situated within a steep, forested valley on the west side of the Queen Charlotte range of mountains, and is less than 700 m from the Pacific Ocean. Skidegate Lake, the only site sampled from

Moresby Island, is located in a forested region, where muskeg and meadow are less prominent.

The Queen Charlotte freshwater locations surveyed represent seven separate drainages, which empty into waters off the northern, western, and eastern shores of the archipelago. The four Argonaut Plain lakes all drain into McIntyre Bay (north of the Graham Island, figure 2): Rouge and Harelda via Damen Creek, and Serendipity and Imber via the Hiellen River system. Drizzle Lake is included within the Skonun River drainage, which also flows into McIntyre Bay. Boulton Lake empties into Kumdis Slough, which is connected with Masset Sound. Mayer Lake (central Graham Island) flows into Mayer Creek through to the Hecate Strait, east of the archipelago. Van Lake drains into Pacific waters west of Graham Island, and Skidegate Lake into Hecate Strait east of Moresby Island via Copper River. Shrimp Lake on the adjacent mainland also drains into Hecate Strait.

B. Collection and storage of specimens

All freshwater stickleback were caught in minnow traps (baited with old cheddar cheese) placed 5 to 10 m from shore on the lake bottom, at a depth of approximately 1 metre. Traps were usually set overnight, although under favourable conditions an adequate number of individuals (20-30) were caught in 2-3 hours during various times of the day. In most locations, gravid females were chosen over juveniles and males, and comprised more than 75% of the samples. Stickleback from Sheldon's Lagoon, however, were collected without regard to sex, and were captured during daylight hours with a small dip net.

Most stickleback were caught between mid May and mid June (table 1), transported live to the University of Alberta, Edmonton, and kept in lake water obtained from their native habitats. Individuals that died either during collection, in transit, or in holding tanks at the university, were first frozen at -20 °C as long as 15 days, then transferred to -70 °C as long as an additional 6 months. Due to reported variable and reduced yields of mtDNA from frozen

tissue (Lansman et al. 1981), fresh samples (killed within 48 hours and kept at 4 °C) were used whenever possible; however, significant mortality of individuals on route and immediately after arrival at the University of Alberta necessitated heavy dependence on frozen samples (approximately 50 %).

C. Laboratory procedures

Extraction of mtDNA from whole fish: All steps were carried out at 4 °C, and solutions were centrifuged in a Beckmann JA20 rotor, unless otherwise stated. This procedure was modified from Lansman et al. (1981) and MacNeil (1986). Composition and preparation of solutions are given in table A1.

Whole stickleback were sliced along the sagittal plane and inspected for parasites; heavy contamination with nematode and cestode worms was encountered, and as some cytoplasmic rRNA genes are highly conserved even across phyla (Moritz et al. 1987), parasite mtDNA could have confounded the results. After removal of parasites, the halves were diced into cubes no more than 0.5 centimeters (cm) in thickness, and homogenized in 10 ml of H medium for 20 seconds at maximum setting using a Tecmar model 1577 tissue blender. The homogenate was transferred to a 30 ml Corex tube, and centrifuged at 3000 rpm (1085 g) for 5 minutes to pellet the nuclei and cellular debris. The supernatant was transferred to a new Corex tube by carefully pipetting from the surface, and the nuclei and debris spun down again. This purification step was repeated two to three times depending on the initial size of the stickleback. After the final purifying centrifugation, the supernatant was transferred to a new 30 ml Corex tube, and spun at 10,000 rpm (20200 g) for 30 minutes to pellet the mitochondria. After discarding the supernatant, the pellet was vigorously resuspended in 4 ml of TE by vortexing for several minutes. SDS was added to a final concentration of 1-2 %, and the solution gently mixed; lysis of mitochondrial membranes was facilitated by first incubating

the solution gently mixed; lysis of mitochondrial membranes was facilitated by first incubating the solution at room temperature for 30 minutes, then overnight at 4 °C.

Proteins, lipids, and other contaminants were removed by several phenol extractions followed by a single phenol-chloroform and a final chloroform extraction. DNA was precipitated by adding 1/3 volume of 7.0 molar ammonium acetate and 2 volumes of 95 % ethanol. After approximately 12 hours at 4 °C, the solution was centrifuged at 10,000 rpm for 30 minutes to pellet the precipitate. The supernatant was discarded and the pellet was rinsed in 70 % ethanol to remove salts and other contaminants before desiccating for approximately 1 hour. The pellet was redissolved overnight in 50 microlitres (ul) of TE. As many samples failed to restrict with different enzymes at this stage, all were further purified with Elutip-d columns in strict accordance with instructions specified by the manufacturer, Schleicher and Schuell.

The final concentration of DNA, as estimated by comparing fractions of the sample electrophoresed through agarose gels and stained with ethidium bromide to known quantities of lambda marker DNA, varied considerably from approximately 200 ng to between 10-100 ug, the vast majority of which was nuclear DNA; mtDNA bands on gels, visualized by ethidium bromide staining techniques, were usually completely masked by nuclear DNA contamination.

Restriction of DNA: Samples were restricted with eight 6-base enzymes (*BglI*, *Eco*RI, *Hin*dIII, *PstI*, *Pvu*II , *SstI*, *Sst*II, *SalI*), one 4-base enzyme (*Hin*fI), and one multiple-base enzyme (*Hin*cII), all supplied by BRL (Bethesda Research Laboratories). The conditions used were those specified by the manufacturer, but with the following additional measures: 1) reactions were carried out in 50 ul total volume to dilute contaminants in solution, 2) samples were digested for up to 4 hours, and 3) spermine was added to a final concentration of 2.0mM to

those samples which only partially digested (Perbal 1984). Even after these reaction promoting steps, 11 samples failed to restrict with *Sst*II, two with *Hin*dIII, one with *Eco*RI.

Double and partial digest procedures (see Maniatis et al. 1982) were employed to map restriction site positions of threespine stickleback mtDNA. For enzymes using compatible React buffers (affording greater than 70% endonuclease activity), double digestions were performed by adding the appropriate amount of both enzymes simultaneously to mtDNA solutions. When reaction conditions required by each enzyme were not compatible, DNA samples were restricted with a single enzyme and purified by phenol-chloroform extraction and subsequent precipitation as described above. The purified fragments were then digested with the second enzyme.

Partial digestions of cloned and fish mtDNA were carried out using a method incorporating serial dilution of enzymes described in Maniatis et al. (1982), over a range of enzyme concentrations of 0.001 to 0.5 units per ug DNA; the most favourable partialling conditions occurred at between 0.005 and 0.01 units of enzyme per ug DNA.

Molecular Cloning: Approximately 300 mg of eggs (extracted from a single gravid female threespine stickleback collected from Hasse Lake, Alberta) were homogenized on ice in 1 ml of H medium using a 2 ml glass hand homogenizer. The homogenate was transferred to a 1.5 ml eppendorf tube, placed in a 20 ml Corex tube, and spun at approximately 3000 rpm (1080g) in a Beckmann JA20 rotor for 3 minutes to pellet nuclei and cellular debris. The supernatant was then transferred to a clean eppendorf tube and spun at 10,000 rpm in an eppendorf centrifuge at room temperature for 30 minutes to pellet mitochondria. After discarding the supernatant, the pellet was redissolved in 150 ul of TE by vortexing for 1 to 5 minutes. SDS was then added to a final concentration of 1%, and the solution kept overnight at 4 °C. DNA was purified and concentrated as described above for the extraction of mtDNA

from whole fish. Three hundred milligrams of stickleback eggs yielded approximately 50 ng of DNA, estimated as for fish DNA yields described above. In contrast to whole fish DNA extractions, mtDNA was more prevalent than nuclear DNA, as evidenced from comparisons of what were presumed to be mtDNA *Eco*RI bands, and background nuclear DNA.

Twenty five ng of the resulting DNA solution and an equal amount of plasmid vector pUC19 (Sambrook et al. 1989) were digested with the endonuclease *Eco*RI. The enzyme and buffer were then removed from both solutions by phenol-chloroform extraction and subsequent ethanol precipitation as outlined above. Stickleback DNA was then ligated to pUC 19 DNA at the *Eco*RI site of the plasmid polylinker following conditions specified by the manufacturer of the of the DNA ligase, BRL: equal amounts of pUC19 and stickleback DNA (10 ng) and 1 unit of T4 DNA ligase were incubated overnight at 4 °C in a 1 X concentration of the React buffer supplied.

Recombinant plasmids were then incorporated into competent DH5 F' *Escherischia coli* cells by transformation procedures described by Glover (1985), and outlined as follows. Two hundred ul of DH5 F' cells stored at -70 °C were thawed at room temperature and left on ice for 30 minutes. Twenty microlitres of DNA solution (or approximately 25 ng of plasmid-insert DNA) were then added to the DH-5 F' competent cells, mixed gently, and kept on ice for 30 minutes. Cells were then heat shocked by placing tubes in water at 42 °C for 90 seconds, then immediately on ice. SOC solution was added and the mixture incubated at 37 °C for 60 minutes. Cells were pelleted by brief centrifugation (3 seconds) in an eppendorf centrifuge, and the upper 750 ul of supernatant discarded. After adding 10 ul of 20 mg/ml X-gal(5-bromo-4-chloro-3-indolyl-b-d-galactoside), the cells were transferred to L plates containing ampicillin at a concentration of 50 ng/ml agar. As ampicillin resistance was conferred by genes on the pUC 19 plasmid, only transformed cells grew.

Colonies from cells containing inserts were differentiated from those with only recircularized plasmids by colony colour. The plasmid polylinker is located within a portion of a gene fragment for B-galactosidase, which is capable of intra-allelic complementation with a defective gene for B -galactosidase of the host (Maniatis et al. 1982). In the presence of galactose (an inducer of B galactosidase production) and X-gal indicator, colonies descended from bacteria with intact B-galactosidase gene fragments (lacking inserts) were light blue. Colonies descended from bacteria with plasmids possessing inserts were white as functional B-galactosidase production was inhibited by the presence of insert DNA.

White colonies were selected and cultured overnight at 37 °C in 5 ml of L broth containing 50 ug/ml ampicillin. Cloned DNA was Isolated from *E. coli* using the alkaline lysis method outlined in Maniatis et al. (1982). Eppendorf tubes containing 1.5 ml of culture were centrifuged at 10,000 rpm in an eppendorf centrifuge at room temperature for 1 minute, and the supernatant removed by aspiration. The pellet was then resuspended in a 100 ul solution of ice-cold GET, and kept at room temperature for 5 minutes. Two hundred microlitres of freshly prepared 0.2 NaOH/1%SDS were added and the solution mixed by inverting the tube several times; the mixture was stored on ice for 5 minutes. Potassium acetate solution (150 ul) was added, and the solution gently vortexed for 10 seconds; the mixture was again stored on ice for 5 minutes. After centrifugation at room temperature for 5 minutes, the supernatant was transferred to a fresh tube, phenol-chloroform extracted to remove proteins and lipids, and the DNA ethanol precipitated as described previously. Inserts were analyzed by comparing restriction fragment patterns of cloned DNA with those of mtDNA isolated from threespine stickleback eggs or whole tissue (see appendix). One clone (pGAMT1) contained the entire mtDNA molecule (see appendix and figure A1.), and was used exclusively to identify mtDNA bound to membranes.

Electrophoresis: Electrophoresis of DNA fragments was performed in horizontal 1.0 % agarose slab gels (11 X 20 X 0.6 cm) in a solution of 1 X TBE buffer. Approximately 3 volts of constant current per centimetre gel length was applied for 16 to 20 hours.

DNA size estimation: Fragment lengths were estimated by comparing the relative mobilities of mtDNA and marker fragments of known size (produced by digesting lambda DNA with *Pst*I and *Hin*dIII). Fragment sizes were determined directly from autoradiographs using the programs SIZER and SIZER2 written by R. N. Beech (University of Alberta, Department of Zoology).

Southern blotting to nylon membranes: With the exception of a few modifications, DNA was blotted to Genescreen Plus as specified by the manufacturer of membranes (Dupont). After staining with ethidium bromide, gels were immersed in 0.2M HCl with gentle agitation for 15 minutes to partially depurinate the DNA. Gels were transferred to a solution of 0.6 N NaOh/ 0.4 N NaCl for 30 minutes to nick and denature DNA, then neutralized in 0.8 N NaCl/0.2M Tris-HCl (pH 8.0) for 30 minutes. Genescreen Plus was prepared by first immersing the membranes in distilled and deionized water, then in 10XSSC for 15 minutes. Two pieces of Whatman filter paper were soaked in 10XSSC, and transferred to a clean, smooth surface. Gels were then positioned squarely on the filter paper, and the convex side of genescreen plus placed on the gel. Two pieces of dry filter paper were placed on top of the screen, followed by a 5 cm thick stack of paper towels and a flat surfaced glass weight. After 30 minutes, the soaked towels in contact with the filter paper were removed, and a stack of dry towels and weight returned. Blots were taken apart after approximately 16 hours. After separation, membranes were immersed immediately in 0.4 N NaOH for 30-60 seconds, to ensure denaturation of DNA, then neutralized in 2XSSC\0.2M tris (pH 8.0). Membranes were then

dried at room temperature overnight or at 65 °C for 2-3 hours. To reduce potential background problems, membranes were boiled for 20 minutes in a solution of 1 % SDS and 0.1XSSC prior to prehybridization.

Radioactive labelling of DNA: Probe DNA was made radioactive using random primer labelling techniques given by the manufacturer of the labelling kit (BRL). Twenty nanograms of clone DNA (pGAMT1) and 5 ng of lambda DNA dissolved in 20 ul of TE were denatured by heating in boiling water for 5 minutes; the solution was then immediately put on ice. While on ice, 2 ul each of the three nucleotides dATP, dGTP, and dTTP [0.5 mM]; 15 ul of random primer buffer solution (containing 18 OD_{260} units/ml oligodeoxyribonucleotide primers, hexamer fraction); 5 ul (approximately 50 *u*Ci) of a-^{32}P, 3000 Ci/ mmol; and 3 ul of distilled water were added to a total volume of 49 ul. The solution was gently mixed, and 1 ul (3 units) of Klenow (large fragment of DNA polymerase I) added and again mixed. The solution was incubated at 25 °C for 3 hours. Labelled DNA was passed through a column of G 50 sephadex to remove unincorporated nucleotides, and denatured by adding 5 N NaOH to a final concentration of approximately 0.2 N. After about 5 minutes, 2 M Tris-HCl (pH 8.0) was added to a final concentration of 0.5 M to neutralize the solution.

Hybridization conditions: Membranes were prehybridized by immersing them in 20 ml of hybridization solution (Church and Gilbert 1984) at 65 °C for a minimum of 1 hour. Denatured labelled DNA and 2 ug of denatured salmon sperm DNA were added to the hybridization solution and massaged over the membranes. Hybridization reactions were carried out at 65 °C under constant agitation for between 24 and 48 hours in sealed ziploc plastic bags.

Labelled DNA (mitochondrial and lambda marker) not hybridized to homologous membrane bound DNA was removed to reduce background signal by first washing membranes in 2XSSC at room temperature for 5 minutes, then twice in a solution of 1%SDS/2XSSC at 65 °C for 45 minutes, and again in 0.1XSSC at room temperature for 5 minutes.

Autoradiography: After washing, membranes were sandwiched in filter paper to blot away excess moisture, and wrapped in cellophane plastic. X-Ray film and membranes were placed in cassettes fitted with Dupont intensifying screens, and kept at -70 °C for between 4 hours and 1 week (depending on the strength of the signal), prior to developing.

D. Analysis of data

Except for *Hin*fI recognition sequences, restriction sites associated with fragments more than 500 base pairs in length were mapped to ensure homology of DNA being compared; fragments less than 500 base pairs in length were not resolvable consistently and therefore were neither scored nor mapped. *Hin*fI restriction sites were not mapped due to the large number of small fragments (greater than 15 below 500 base pairs) generated by the enzyme.

Composites of restriction site patterns produced by the nine enzymes for which sites were mapped, defined the haplotypes. The number of nucleotide substitutions per nucleotide site ($\hat{d}$) was estimated from restriction site information using equation 28 from Nei and Tajima (1983). Since *Hin*fI recognition sequences were not mapped, data obtained from this enzyme were excluded from calculations of nucleotide divergence. However, the number of inferred site differences between *Hin*fI fragment patterns were presented with all major results based on mapped site differences.

Proportion of nucleotides differing between homologous sequences (π) was originally

calculated using equation 1 from Nei and Tajima (1983), to compare the divergence observed here (given as substitutions per nucleotide site) to amounts documented in other studies, which were frequently expressed as percent nucleotide diversity. At lower levels of divergence, δ and π values are quite similar, as the likelihood of two substitution events occurring at a specific site is quite small. In this study, δ values were within about 1.5% of π values, therefore, only the former (δ) was presented and used in direct comparisons with π given in other reports.

Site presence\absence states were used to construct a hand drawn parsimony network relating haplotypes. The UPGMA (Unweighted-Pair-Group Method using Arithmetic averages) was used to cluster divergence estimates of the 12 mtDNA haplotypes.

III. Results

In this study mtDNA differences amongst freshwater and marine threespine stickleback were surveyed to reconstruct historical times and patterns of colonization within the QCI, and to provide clues about the evolution of several morphologically distinctive populations. Mitochondrial DNA was characterized using methodologically expedient restriction enzyme techniques; restriction enzymes cleave double stranded DNA at specific recognition sequences producing a series of fragments, the size and number of which depending on where along the DNA molecule the sites are located. This method of analysis revealed two types of variation between mtDNA sequences: restriction site and DNA length differences. A restriction site change often appeared as a decrease or increase in the number of fragments observed, but the sum of the fragment lengths in the two patterns compared remained about equal. For a restriction site loss, a single larger fragment was generated from two smaller fragments of an equivalent combined length. A restriction site gain produced two smaller fragments from a single, larger fragment. So, from comparisons of restriction length patterns, restriction site differences between two mtDNA types could have often been inferred. Occasionally, however, molecules with a different arrangement of restriction sites can generate phenotypically similar fragment patterns. If the slight length differences between these fragment patterns are beyond the resolving power of the methods employed, then the similarly sized fragments may be identified as homologous. In such instances, analyses using inferred restriction site changes would produce less accurate results. By mapping restriction site locations and using actual site changes, many of the limitations inherent in methods using comparisons of restriction fragment patterns or inferred restriction site differences, were avoided.

Large discrepancies (+100 base pairs) in the fragment length sums of different restriction fragment patterns were taken as evidence of DNA length as opposed to site variation. This was confirmed by restricting a suspected length variant type individually with

several enzymes, and mapping the novel fragment generated in each digest. In all instances, the atypically lengthed fragment spanned a common region of the mitochondrial genome, presumably the location where the insertion/deletion event occurred. In addition, the departures of each of these fragments from the more typical lengths (observed in digests of other mtDNA's with respective enzymes) were about equal.

A. Restriction fragment and site variation

All ten restriction enzymes cut stickleback mtDNA at least twice, and eight revealed fragment length polymorphisms attributable to restriction site differences (figure 3, table A2). *Hinc*II and *Hin*fI enzymes revealed extensive variation, both producing seven different restriction fragment length patterns (table A2), with some profiles having as few as two of ten bands in common (figure 4). Most of the other six base polymorphic enzymes yielded either two or three different restriction fragment length patterns. Combining restriction fragment patterns produced by all ten enzymes, 20 composite digest profiles were identified (table A3).

Excluding the tetranucleotide *Hin*fI recognition sequences, 40 restriction sites were surveyed (representing 1.4% of the 16.6 kilobase mitochondrial genome), of which 18 were polymorphic (figure 5).

B. Length variation

Based on averages of the sums of fragment lengths produced by each of the eight hexanucleotide enzymes, more than 95% of all mitochondrial genomes were 16.6 kilobase (+/- 50 base pairs). The seven variants detected were increases with respect to the common genome size, and were restricted to three populations and two closely related haplotypes. The genomes of four stickleback from Skidegate Lake (1, 7, 10, and 11, see table A3), all type (H) as defined on the basis of restriction site information, were 16.75 kb (kilobase). Similarly, the

two large genomes identified from Mayer Lake, (17.13 kb in individual 372 and 16.88 in 368) were also type (H). The length increase identified in Skidegate Lake (400 base pairs) was from an individual with type (I) mtDNA, which is the most similar to haplotype (H), separated by a single restriction site.

Length variation originated from within an approximately 800 base pair region between the *Hin*cII site at position 51 and the *Bgl*I site at position 56 (figure 5). This was inferred from length polymorphisms (not attributable to site changes) observed in the 3.70 *Hin*cII fragment flanked by restriction sites at positions 35 and 56, and the 5.71 *Bgl*I fragment flanked by sites at positions 51 and 86. In addition, some of the greater genome length increases were also detected in the larger (>8.50 kb) *Eco*RI, *Hin*dIII, *Pst*I, *Sst*I, and *Sst*II fragments encompassing the region 51 to 56; the lesser genome length increases were about 1 % of the size of these larger fragments and therefore not resolvable. No length differences attributable to genome size variation were observed in any of the smaller fragments generated by these enzymes outside 51-56 (spanning 65 to 22, and 32 to 43, figure 5).

C. Heteroplasmy

A single instance of site heteroplasmy (the occurrence of more than one mtDNA type in a single individual) was observed. Individual 306 from Boulton Lake exhibited patterns *Pst*I(c) and *Bgl*I(e), composites of patterns *Pst*I(a) & *Pst*I(b), and *Bgl*I(c) & *Bgl*I(d), respectively (table A2). No instances of genome length heteroplasmy were detected.

D. Geography of haplotypes

Based on differences in mapped restriction sites, 12 mtDNA haplotypes were identified (figure 6), 11 from the west (A-I and K & L), and one from the east coast of Canada (J). Haplotype (A) was observed in marine stickleback and all freshwater localities, except Van

and Rouge lakes (table 3). Haplotypes (C) and (E) were also found in marine stickleback, and while (E) was exclusively marine, (C) was identified in most Argonaut Plain populations from northeast Graham Island, and Van Lake from southwest Graham Island. Haplotypes (B) and (H) were not observed in marine stickleback but were present in several freshwater populations separated by marine waters. Both types (D) and (G) were confined to Boulton Lake, and (F), (I), and (K) to Van, Skidegate and Imber lakes, respectively.

E. Parsimony relationship amongst haplotypes

Haplotypes from the coast of British Columbia grouped into two distinct assemblages: the pacific and rouge lineages (figure 6). The pacific lineage was represented by nine haplotypes (A-I) interrelated by single site differences, except (G) which was separated from the nearest by two restriction sites. Haplotype (A) was at the centre of a parsimony network relating the assemblage; five other types (B, C, D, E and G) connect separately with (A), and all but (G) by a single site difference. In the longest path within the lineage, (I) is connected with (B) through (H). The rouge lineage was comprised of two haplotypes (K and L), separated by a single site change.

Rouge and pacific lineages were interderivable by a minimum of seven restriction site changes. This comparatively large genetic break was apparent also in *Hin*fI restriction fragment profiles. *Hin*fI pattern (g), characteristic of all rouge lineage stickleback (figure 6), was a minimum of three restriction site differences from the most phenetically similar pattern (a), by far the most common profile associated with pacific lineage haplotypes. The five remaining *Hin*fI fragment profiles (b-f), each observed in no more than three individuals, were a single inferred restriction site from (a).

In the most parsimonious relationship between the atlantic haplotype and the pacific lineage, (J) is interderivable from the closest haplotype, (I), by three site changes. Haplotype

(J) exhibited *Hin*fI restriction profile (a), exemplifying the greater similarity between atlantic and pacific versus pacific and rouge lineage mtDNA.

The UPGMA phenogram generated from δ values (figure 7) resembles the phylogeny constructed from presence-absence restriction site information (see figure 6). The distinction between rouge lineage and remaining haplotypes is apparent, with the two assemblages separated by about 0.028 substitutions per base pair. The conflicting placements of (J) represents the only major incongruity between the two figures. An alternative relationship with (J) connecting directly with (A) (see figure 8) requires only a single additional site change, and is more in agreement with phenogram depicted in figure 7.

F. Divergence between freshwater and Pacific marine Stickleback

In addressing the historical colonization of the QCI, I focused on the extent of mtDNA divergence between lacustrine populations and nearby marine stickleback, the putative ancestor of freshwater stickleback in the area. No haplotypes other than those observed in marine samples were found in Drizzle Lake (table 3). Haplotypes observed in Van, Boulton and Mayer lakes were separated by no more than two restriction site differences (fewer than 0.0060 substitutions per base pair) from those identified in marine stickleback (table 3). In Skidegate Lake, the most divergent haplotype (I, found in only a single individual) was distinguishable from marine haplotypes by three restriction sites (or 0.0087 base substitutions). The four Argonaut Plain populations (Harelda, Imber, Rouge and Serendipity) located on the northeast corner of Graham Island, were represented mostly by individuals with (L) haplotypes, interderivable from those observed in marine stickleback by no fewer than eight restriction site changes (0.0245 base substitutions). Moderate to high frequencies of the common form (L) within these four adjacent localities (table 3), yet complete absence from marine and all other freshwater populations surveyed (including the lack of all eight intermediaries between

(L) and the nearest marine haplotype) argues against its occurrence in marine stickleback.

The extent of divergence from Pacific marine samples observed in populations south and east of the Argonaut Plain (see table 3) is comparable to that reported for Shrimp Lake on the adjacent mainland (0.0056 base substitutions), which was colonized after deglaciation of the west coast of North America (approximately 10,000 years B.P., Heusser 1960). The four Argonaut Plain populations, however, exhibit much greater sequence divergence from Pacific marine stickleback, indicative of perhaps a much earlier separation. In fact, some haplotypes from the Argonaut Plain were more differentiated from Pacific marine types than were those surveyed from Nova Scotia (0.0115 base substitutions), 16,000 km distant and within an entirely separate ocean basin.

Given the phylogeography of haplotypes observed, maximum divergence estimates of many non-Argonaut Plain populations from marine stickleback may be somewhat inflated. Haplotype (H) was observed in three widely disjunct freshwater localities: Shrimp Lake on the adjacent mainland; Skidegate Lake on Moresby Island; and Mayer Lake on Graham Island (figures 1 & 2). Either the two restriction sites separating (H) from the nearest haplotype observed in marine populations (A), evolved in parallel in all three lakes, or the haplotype was common in the source gene pool (presumably marine populations of stickleback) from which primary and/or secondary introgression into QCI and adjacent mainland populations must have occurred. The apparent absence of (H) from marine populations surveyed could be owing to somewhat limited sampling efforts (20 marine stickleback were analyzed), or a recent decline in the frequency of the haplotype. This argument is applicable also to haplotype (B), intermediate between (A) and (H), and found in two widely disjunct freshwater localities (Imber Lake, north Graham Island, and Skidegate Lake, Moresby Island). Given the occurrence of (H) and (B) haplotypes in marine waters (either presently or in the recent past), most non-Argonaut Plain freshwater populations have diverged little from marine stickleback

(fewer than an estimated 0.0030 base substitution differences, table 3). Only in Boulton Lake were greater amounts of sequence divergence from marine stickleback observed (0.0056 base substitutions).

G. Phylogeography of haplotypes in relation to stickleback morphology

Included within the QCI freshwater populations surveyed were many stickleback morphotypes highly divergent from the common marine or freshwater forms. Several of these types were not confined to specific sites: some were observed in several lakes within a given drainage and others in separate watersheds. If allopatric stickleback populations of a particular morphotype were not independently derived from marine stickleback following deglaciation of the QCI, but rather were descended from a common freshwater relic ancestor (possibly of similar morphological constitution), then mtDNA patterns should be more similar amongst these freshwater populations than between *leiurus* and *trachurus* stickleback. I report here findings consistent with the recent and independent evolution of a particular morphotype (occurring in several sites) from marine stickleback, but also possible monophyly for other morphologically similar stickleback occurring in four populations from two separate watersheds.

Giant, melanistic stickleback occur in Drizzle and Mayer lakes, both located on Graham Island, and within adjacent watersheds. Mitochondrial DNA haplotype (A), common in marine samples, was fixed in Drizzle Lake. In Mayer Lake, (H) was most frequent, followed by haplotype (A). Regardless of whether (H) is present in marine stickleback, these two freshwater populations are at least as differentiated from each other as is either from *trachurus*. Within Skidegate Lake, located on north Moresby Island, are giant silver stickleback. As in Mayer Lake, haplotypes (A) and (H) were both common, but (B) and (I) were also identified, though at lower frequencies. Assuming for a moment the absence of

haplotype (H) in marine stickleback, its predominance in both lakes (table 3) could be interpreted as evidence for a common freshwater ancestry, possibly from a morphologically similar stickleback population. These two lakes, however, are on different islands (Mayer on Graham Island and Skidegate on Moresby Island), so the scenario would require the movement of freshwater fish across Skidegate Inlet or Skidegate Channel. Furthermore, haplotype (H) was observed also in stickleback from Shrimp Lake on the adjacent mainland, separated by more than 80 km of saltwater. And Shrimp Lake stickleback do not resemble the giant forms, but rather are much more morphologically similar to the common freshwater type on the west coast of North America. Given the more parsimonious interpretation to the patterns of mtDNA variation observed, where haplotype (H) in Mayer, Skidegate and Shrimp Lakes is presumed to reflect a marine rather than freshwater ancestry, all three giant populations have evolved quite recently, perhaps from *trachurus*.

The four Argonaut Plain populations were all characterized by lateral plate loss, and marked spine reduction. A highly divergent lineage of haplotypes (the rouge lineage), at least seven sites removed from the pacific lineage, was also common to all four populations, and completely fixed in one (table 3). The absence of these divergent haplotypes, (and all intermediaries) in marine population(s) would implicate introgression from a common ancestral source long separated from marine stickleback, presumably within or nearby northeast Graham Island. In addition to a similar mtDNA composition, these hypothetical ancestral stickleback also may have been morphologically allied to the Argonaut Plain stickleback surveyed.

Some Boulton Lake stickleback were exceptional amongst non-Argonaut Plain freshwater fish in exhibiting loss or reduction in spines and pelvic girdles. Exclusive to the Boulton population is mtDNA haplotype (G), which shares a restriction site with those of the rouge lineage but with no other type observed in the pacific lineage. Haplotype (G), however, is still separated by a minimum of seven restriction sites (0.0219 base substitutions) from the

nearest of the rouge lineage, and only two restriction sites (0.0560 base substitutions) from another pacific lineage haplotype. Thus, this association between morphology and mtDNA is limited.

IV. Discussion

A. Length variation

Mitochondrial DNA length variation of the magnitude observed here has been reported for several fish species, including *Amia calva* (Bermingham et al. 1986), members of the family Scorpaenidae (Beckwitt & Petruska 1985), *Stizostedion vitreum* (Billington & Hebert 1988), *Culaea inconstans* (M. Gach, personal communication), and *G. aculeatus* (Gach & Reimchen 1989). The location from which length increases originated (51-56, figure 5) roughly coincides with regions associated with genome size variation (and often identified as the D loop) mapped in other species. In *Stizostedion vitreum* for example, length variation was reported to have originated from a location approximately 1.5 kb from the nearest of two widely conserved *Sst*II sites (see Billington and Hebert 1988); this corresponds to approximately positions 53 or 33 in the threespine stickleback mtDNA restriction site map.

Genome size variation appears to be associated with mtDNA type as defined on the basis of restriction site information; mitochondrial DNA molecules departing from the typical 16.6 kb length (observed in seven individuals from three locations) were either (H) or the closely related (I) types. Often, discrete length variations arise from differences in copy number of short tandemly repeated sequences (Densmore et al. 1985; Harrison et al. 1985), usually restricted to control regions (Moritz et al. 1987). The approximate length increases of 150, 275, 400, and 525 base pairs observed here could be owing to the occurrence of a variable number of copies of a 125 to 150 base pair repeat, perhaps specific to (H) and certain related haplotypes.

B. Heteroplasmy

More than one type of mtDNA was observed in a single fish from Boulton Lake.

Although generally rare, heteroplasmy has been reported also in dairy cattle (Hauswirth and Laipis, 1982; Olivo et al., 1983), lizards (Densmore et al. 1985), and crickets (Harrison et al. 1985). This condition may arise through mutation or parental contribution (Wilson 1989). Alternatively, the apparent co-presence of two haplotypes within a single stickleback observed here may be due to contamination of a Boulton Lake sample with tissue from one or more other fish. Since the entire animal was used in the analysis, exclusion of experimental error as a potential cause was not possible.

C. Diversity of haplotypes

Considering the amount and direction of sampling effort expended, mtDNA diversity reported here is well within the range documented elsewhere for various fish taxa (table 4). In many of these studies fewer individuals were analyzed, but samples were collected over a much broader area. As phylogenetic divergence is expected to increase with increasing geographic distance between populations, the effect of sampling fewer individuals in contributing to the total number of haplotypes observed was likely offset by sampling from more distant localities. Also, in most studies a greater proportion of the mitochondrial genome (about 40-100% more nucleotides) was surveyed, thus allowing the detection of a comparatively greater proportion of existing diversity.

Intrapopulation haplotype diversity varied considerably amongst localities (table 3). As many as five haplotypes were observed in Imber, while Rouge and Drizzle populations were monotypic; Gach and Reimchen (1989) also observed only a single mtDNA type in Drizzle Lake. This variation does not seem to be explicable by the slight differences in sampling effort (table 3). The lack of diversity observed in Rouge Lake could be due to recent bottlenecks as the lake is less than 2.0 ha (hectares) in area; however, Drizzle Lake is quite large (approx. 112 ha) and has an estimated population size in excess of 1.0×10^4 (T.

Reimchen, presonal communication). Perhaps these populations were colonized by relatively few individuals, and subsequent introgression from marine stickleback was minimal. Alternatively, the lack of variation may reflect homogeneity within the source populations.

D. Extent of temporal habitation and evidence for QCI refugia

Patterns of mtDNA variation observed here are consistent with a post-glacial origin of most of the freshwater populations surveyed from marine fish. Some individuals from the four Argonaut Plain localities, however, appear to have descended from freshwater populations which last shared a female common ancestor with marine stickleback prior to the Wisconsin. Freshwater stickleback outside the Argonaut Plain exhibited little mtDNA sequence divergence from marine stickleback in the surrounding Pacific Ocean, comparable in fact to amounts estimated for Shrimp Lake on the adjacent mainland, which presumably was colonized after the retreat of the Cordilleran ice sheet (approximately 10,000 years B.P.). For the most part, these populations were comprised of a group of closely related haplotypes also observed in marine samples. The phylogeography of some haplotypes that were specific to freshwater localities strongly suggests their occurrence in marine stickleback as well. Haplotypes a single restriction site (approximately 0.0027 base substitutions) removed from those observed or inferred to occur in marine stickleback were found in Skidegate and Van lakes. Given a rate of mtDNA sequence divergence of approximately 0.02 substitutions/base pair/10^6 years, (estimated for primates, Brown et al. 1979, 1982), even fewer differences (approximately 0.0003 substitutions) between lake and marine stickleback mtDNA were expected under a post-Pleistocene scenario of colonization. However, considering the amount of variance associated with estimates of divergence based on only a few restriction site differences (see table 2), this discrepancy is probably not important. Two restriction sites (or 0.0546 base substitutions) separated haplotype (G) in Boulton Lake from marine haplotypes. Although this

in itself is not very compelling evidence for an earlier separation of the population from marine stickleback, among the non-Argonaut Plain stickleback in Boulton Lake individuals only are found 1) mtDNA with restriction site P_1 (figure 5 & 6), common to rouge haplotypes but found in no other from the pacific lineage, and 2) the occurrence of a highly distinctive morphotype similar to forms observed in some of the Argoanut Plain lakes surveyed but rarely observed elsewhere. In other words, the non-Argonaut Plain population with mtDNA which has differentiated the most from marine types, is also genetically and morphologically most similar to a group of populations for which there is more substantive evidence of an even longer period of isolation from marine stickleback.

In nearly half (46 %) of the individuals surveyed from the Argonaut Plain, rouge lineage mtDNA was identified. If the lineage did originate within freshwater stickleback, the extent of divergence from haplotypes observed in marine samples should be indicative of the length of habitation of freshwater environments in the area by the species. Rouge lineage mtDNA from the four Argonaut Plain populations was considerably more divergent from Pacific marine mtDNA (δ =0.0245) than were haplotypes from all other stickleback populations surveyed, including the freshwater site from the adjacent mainland, and the single Atlantic Ocean locale (table 3). The time of separation of Pacific and Atlantic Ocean stickleback is not known, but at present 8000 km of Arctic waters separate the most northerly extant populations (distances determined from stickleback distributions given by Wootton 1984).

The extent of divergence between rouge lineage stickleback from the Argonaut Plain, and Pacific marine stickleback (only several kilometres distant) is comparable to or greater than maximum amounts of intraspecies divergence calculated in most other studies of fish mtDNA phylogenetics, often conducted over extensive geographical ranges. (table 4). The larger levels of divergence reported by Bermingham and Avise (1986) for *Lepomis gulosus* and *L. microlophus* (6.3 & 8.7 % sequence divergence, respectively) were for populations from

south central versus south eastern continental United States. It was suggested that vicariant events during the Pliocene (2-4.5 X10^6) may have initiated these major genetic breaks.

In using the approximately 0.02 substitutions/base pairs/10^6 years rate of mtDNA sequence divergence adopted in most mtDNA surveys of fish (Avise et al. 1984; Thomas et al. 1986; Avise et al. 1987b; Avise and Vrijenhoek 1987; Billington and Hebert 1988; Gonzalez-Villasenor and Powers 1990), the proposed freshwater refugial population(s) from the Argonaut Plain separated from Pacific marine stickleback approximately 1.34X10^6 years B.P. (table 5). DNA polymorphisms in the ancestral population make this an overestimate of the time of divergence. Assuming that the amount of polymorphism in the ancestral population (δ_A) can be estimated by $\delta_A = 0.5\ (\delta_x + \delta_y)$ (from Nei and Li 1979; Wilson et al. 1985), where δ_x and δ_y are mean pairwise mtDNA divergence values between individuals in population x and y, respectively, the corrected estimate is approximately 1.25X10^6 years B.P. (table 5). Given the large variances of divergence estimates (table 2), and the use of rates of mtDNA evolution calibrated for taxa distantly related to threespine stickleback, this estimate is provisional. However, even if correct within an order of magnitude, populations of freshwater stickleback within the QCI were isolated from marine stickleback prior to the Wisconsin, and have persisted through to the present. This also is suggested by relative times of separation inferred from comparisons of divergence with: 1) other freshwater populations in the area, 2) stickleback from the Atlantic Ocean, and 3) that observed in surveys of mtDNA variation in other fish species.

Radiocarbon dates obtained for two of the four Argonaut Plain lakes (Rouge and Serendipity) do not indicate pre-glacial origins of either site (Warner 1984; R. Mathewes, personal communication). Nearby locations within the Hiellen River drainage (figure 2), surrounding Argonaut Plain, or possibly formerly exposed shelves to the east in Hecate Strait, may have provided source populations from which these sites were colonized after

deglaciation.

E. Phylogeography of haplotypes in relation to stickleback morphology

In addressing the origin of morphological endemism in QCI stickleback, Moodie and Reimchen (1976a) concluded that distinct populations evolved in situ from marine stickleback following deglaciation (see also Reimchen et al. 1985). This implies that characteristics common to several allopatric freshwater populations, and absent in *trachurus*, evolved in parallel.

The phylogeography of haplotypes observed here is consistent with the independent origin and parallel evolution of gigantism observed in several allopatric populations, but indicates a possible historical component to other morphological characteristics observed in several freshwater locations. Lateral plate loss and major spine reduction, observed in stickleback surveyed from the Argonaut Plain, was very rare amongst the approximately 120 QCI lakes surveyed for morphological endemism (see Moodie and Reimchen 1976b; Reimchen 1984), and has been reported elsewhere in North America only from Texada Island, British Columbia (J. McPhail, personal communication), and several lakes in California (Miller 1960). A potential historical component to these morphological similarities (i.e., common ancestry) would be implicated if the Argonaut Plain stickleback belonged to a phylogenetic branch long separated from marine mtDNA. Mitochondrial DNA types (K) or (L), 0.0209-0.0245 base substitutions from the nearest haplotype observed in the study, were found in all four populations, but not elsewhere within the remaining sites surveyed. However, haplotypes (A), (C), and (E) were observed also in Serendipity, Imber and Harelda populations. In other words, not all of the Argonaut Plain stickleback surveyed belonged to this divergent mtDNA lineage.

The co-occurrence of pacific and rouge lineage haplotypes in three of these populations may be a result of recent secondary introgression of *trachurus* into previously existing freshwater populations descended from rouge lineage stickleback. The absence of two discernable morphs (*trachurus* and the postulated divergent Argonaut Plain type), may be a result of hybridization and recombination. If immigration of marine fish was minimal, the original morphotype may not have been swamped completely; because of the small effective population size of mtDNA versus nuclear genes, the incursion of relatively few individuals may have been sufficient to introduce pacific lineage haplotypes into some of these populations.

Large phylogenetic discontinuities of the magnitude observed in Harelda, Rouge and Imber lakes (greater than about 2 percent sequence divergence), have been reported rarely for conspecifics from a single collection site (see Avise et al. 1987a). In a survey of mtDNA variation amongst populations of mummichog *(Fundulus heteroclitus)* along the Atlantic coast of North America, Gonzalez-Villasenor and Powers (1990) identified two distinctive mtDNA haplotypes (p=0.0196) within the Vince Lombardi, New Jersey, collection locality. The only other published example observed was for bluegill sunfish *(Lepomis macrochirus)*, which were sampled from localities throughout much of south eastern continental United States: two grossly divergent mtDNA types (p=0.0850) were reported from Lake Oglethorpe, north Georgia (Bermingham and Avise 1986). In both of these studies a similar historical scenario as suggested here (secondary introgression between two long isolated populations), was proposed to account for the large genetic "breaks". The initial split between the two formerly isolated mummichog populations was estimated tentatively to have occurred approximately $1.0X10^6$ years B.P., and the sunfish groups more than $4.0X10^6$ years B.P.

The founding of the Argonaut Plain populations by morphologically distinct stickleback with rouge lineage mtDNA, and subsequent introgression of fish recently derived

from marine stocks is suggested also by an association between the extent of morphological divergence from the common freshwater or marine forms, and the prevalence of pacific lineage haplotypes, an indicator, perhaps, of the extent of recent secondary introgression. Stickleback in Harelda and Imber lakes lack lateral plates, but of the Argonaut Plain populations surveyed, appear to be the least morphologically divergent from the common freshwater or marine form. The frequency of pacific haplotypes in these two populations was approximately 0.75. In addition to lateral plate loss and spine reduction, stickleback in Serendipity Lake lack all vestiges of a pelvic girdle; the frequency of pacific lineage haplotypes in the population was 0.5. In Rouge Lake, no pacific lineage haplotypes were identified, and fish lacked lateral plates and exhibited the greatest reduction in dorsal and anal spines. Furthermore, stickleback showed almost complete pelvic girdle loss, and were characterized by a post cranial hump, and two dorsal fins.

I did not observe a greater phylogenetic association amongst the giant Drizzle, Mayer and Skidegate stickleback populations, than between any one of these and other morphologically typical freshwater or marine stickleback. Fish from these three lakes were comprised of (H) and/or (A) haplotypes; these types also occurred in other freshwater populations (table 3), and likely marine stickleback as well. The phylogeography of haplotypes and lack of divergence between the giant populations and *trachurus* indicates that these lakes likely were not colonized by morphologically similar relic stickleback. However, the limited resolution of the methods employed here does not permit examination of whether: 1) freshwater populations (i.e., giant Drizzle and giant Mayer Lake stickleback) were independently derived from marine stickleback and evolved in isolation within the last 11,000 years; or 2) freshwater populations were colonized by other post-glacial freshwater stickleback in adjacent watersheds following deglaciation. In other words, although the evidence suggests that the morphological differences between the giant form and *trachurus* have accrued quite

recently (within the Holocene), little can be said as to whether gigantism has evolved independently in both lakes, or whether similarities are owing to primary or secondary introgression between these populations following deglaciation. At a rate of mtDNA divergence of approximately 0.02 substitutions/base pair/10^6 years, fewer than ten base substitutions would be expected between mtDNA lineages separated immediately following deglaciation of the QCI; in surveying only a small portion of the 16.6 kb mitochondrial genome, few (if any) of these differences likely would be detected. In addition, even if a substantially greater proportion of the mitochondrial genome were surveyed, polymorphisms present in the ancestral marine population (if similar to that observed in extant marine stickleback) would make distinction between scenarios involving colonization by marine versus colonization by other freshwater populations within the Holocene, problematic.

Conclusions pertaining to both the existence of pre-Holocene freshwater populations of stickleback, and the possible historical component to morphological similarities (lateral plate loss and spine reduction) observed in some allopatric stickleback populations, were based on the presence of rouge haplotypes within the Argonaut Plain; the lineage was presumed to have evolved within (or nearby) the region in isolation from marine stickleback, supposedly over an extended period of time. Although this seems the most parsimonious interpretation, there are several other alternative explanations to the patterns of mtDNA phylogeography observed. The mtDNA composition of the Argonaut Plain populations may be the result of post-glacial introgression from marine populations comprised of both rouge and pacific lineage haplotypes. Computer simulations indicate that genetic breaks of this magnitude within continuous marine environments may occur through stochastic lineage sorting (Saunders et al. 1986). Perhaps these haplotypes were either: 1), confined to marine waters near the mouth of the Hiellen River, or 2), present on a broader scale but not represented in any of the marine or other

freshwater populations due to limited sampling effort. Limited phylogenetic divergence within ocean waters (p=0.001-0.0100) over an even greater geographic scale reported in surveys of other fish species (reviewed in Avise et al. 1987a; Gonzalez-Villasenor and Powers 1990) argues against either scenario, and patterns of mtDNA variation observed in such geographically remote locations as Masset Harbour (north coast of Graham Island), Van Lake (southwest Graham Island), Sheldon's Lagoon, (east Graham Island), and Shrimp Lake (adjacent mainland), argues against the former.

Alternatively, the four Argonaut Plain populations may have been colonized at the end of the Pleistocene from marine stickleback genetically similar to those observed in the survey, and subsequently diverged at an unequal and rapid rate from stickleback in the Pacific Ocean. Rates of mtDNA evolution varying by as much as three-fold have been observed in different lineages of *Drosophila* (DeSalle and Templeton 1988) and may be attributable to the influence of population size and bottlenecks on the rate of fixation of near-neutral mutations (Ohta 1973, 1976). However, an evolutionary rate approximately two orders of magnitude beyond that estimated for primates would need to be invoked to account for the amounts of sequence divergence calculated between Argonaut Plain populations and Pacific marine stickleback, which, under this scenario, separated at most 11,000 years B.P.

F. Future research

The possibility of a marine origin of the rouge lineage mtDNA could be evaluated further by additional sampling of marine locations, particularly off the Hiellen and Damen drainages, within the McIntyre Bay (figure 2). A complementary phylogenetic analysis of another fish species (i.e., *Cottus asper*) occurring within the Hiellen and other river drainages from Graham Island, may help discriminate between stochastic versus deterministic explanations given for the patterns of mtDNA phylogeography observed. The actual site(s)

postulated to have escaped glaciation might be localized by extending the survey of freshwater stickleback populations to the headwaters of the Hiellen River drainage and other regions within the Argonaut Plain. Genetic surveys of terrestrial species (both plants and animals) within the region would be useful in determining the ecological scope (i.e., aquatic versus aquatic and terrestrial) of the proposed refugium.

The hypothesis of secondary introgression between allopatrically evolving populations forwarded to explain the co-presence of divergent haplotypes in Imber, Harelda, and Serendipity Lakes could be tested by investigating both nuclear and mitochondrial DNA markers in individuals from Rouge Lake, the surrounding Pacific Ocean, and one of the three Argonaut Plain sites comprised of stickleback with both pacific and rouge lineage mtDNA. Additional assays of certain nuclear genes also may contribute to our understanding of the genetics of some of the marked morphological differences observed amongst the QCI stickleback populations surveyed.

Table 1: Date of collection and location of sites surveyed.

Site	Month and year of collection	Latitude and longitude	Specific description of trap locations
Masset Harbour	Oct, 1989	54° 01' 06''N 132° 08' 00''W	
Rouge Lake	June, 1988	54° 02' 00''N 131° 52' 20''W	south and southeast shores
Harelda Lake	June, 1988	54° 01' 45''N 131° 50' 45''W	northeast shore
Serendipity Lake	June, 1988	54° 01' 40''N 131° 45' 20''W	north and northeast shores
Imber Lake	June, 1988	54° 01' 30''N 131° 44' 25''W	southwest shore
Drizzle Lake	June, 1989	53° 56' 00''N 132° 04' 50''W	southeast shore, northwest of lake inlet creek
Boulton Lake	June, 1989	53° 47' 00''N 132° 05' 50''W	east shore, paralleling yellowhead highway
Mayer Lake	June, 1988	53° 38' 20''N 132° 03' 25''W	south and extreme southwest shore
Van Lake	June, 1988	53° 17' 15''N 132° 29' 30''W	northwest shore, north of lake outlet
Skidegate Lake	June, 1988	53° 06' 25''N 131° 56' 35''W	north shore, where lake narrows near old bridge
Sheldon's Lagoon	June, 1988	53° 08' 50''N 131° 45' 20''W	small channel during low tide at northwest corner of lagoon
Shrimp Lake	Aug, 1989	54° 14' 30''N 130° 06' 20''W	northeast shore
Nova Scotia	Nov, 1989	45° 26' N 61° 31' W	Guyysborough Harbour near the town of Glen Keen

Table 2: Pairwise estimates of sequence divergence (δ) X 10^2 between mtDNA haplotypes (above diagonal) and their standard deviations (below diagonal), calculated from formula 28, Nei and Tajima (1983).

Haplotype	A	B	C	D	E	F	G	H	I	J	K	L
A	-	0.284	0.276	0.275	0.284	0.549	0.546	0.564	0.868	1.151	2.091	2.455
B	0.287	-	0.565	0.564	0.583	0.843	0.839	0.284	0.582	1.475	2.452	2.837
C	0.278	0.405	-	0.547	0.565	0.268	0.814	0.841	1.150	1.429	2.374	2.744
D	0.277	0.404	0.392	-	0.564	0.816	0.812	0.839	1.147	1.426	2.368	2.737
E	0.287	0.418	0.405	0.404	-	0.843	0.839	0.867	1.186	1.475	2.452	2.837
F	0.393	0.497	0.270	0.481	0.497	-	1.079	1.114	1.428	1.704	2.653	3.028
G	0.392	0.494	0.480	0.479	0.494	0.552	-	1.108	1.421	1.696	2.019	2.368
H	0.404	0.286	0.496	0.494	0.511	0.572	0.570	-	0.284	1.149	2.734	3.123
I	0.512	0.418	0.591	0.590	0.610	0.661	0.658	0.286	-	0.870	3.123	3.536
J	0.592	0.685	0.662	0.660	0.683	0.724	0.721	0.591	0.513	-	3.417	3.836
K	0.830	0.917	0.886	0.884	0.917	0.939	0.800	0.969	1.057	1.109	-	0.291
L	0.918	1.007	0.972	0.970	1.007	1.023	0.884	1.058	1.150	1.201	0.294	-

Table 3: Maximum sequence divergence estimates (δ) of freshwater and Atlantic populations from Pacific marine stickleback. The haplotype compostion of each location is also given.

Location (N)	Haplotype (frequency)	Divergence from nearest marine haplotype observed +	Divergence from nearest marine haplotype observed or inferred ≠
Rouge (10)	L (100)	0.0245	0.0245
Harelda (16)	A (56) C (13) L (31)	0.0245	0.0245
Serendipity (15)	A (40) C (7) L (53)	0.0245	0.0245
Imber (15)	A (20) B (13) C (47) K (7) L (13)	0.0245	0.0245
Drizzle (12)	A (100)	0	0
Boulton (9)	A (78) D (11) G (11)	0.0056	0.0056
Mayer (11)	A (28) H (72)	0.0056	0
Skidegate (12)	A (33) B (8) H (50) I (8)	0.0087	0.0028
Van (11)	C (9) F (91)	0.0027	0.0027
Shrimp (12)	A (67) H (33)	0.0056	0
Masset (8)	A (38) C (62)	------	------
Sheldon (13)	A (62) C (31) E (8)	------	------
Nova Scotia (3)	J (100)	0.0115	0.0115

N represents the number of individuals surveyed from a given location. The largest of pairwise divergence estimates between haplotypes from a given locality and the most closely related from Pacific marine samples, was used as a measure of the maximum amount of divergence of a population from Pacific marine stickleback. Estimates were calculated between freshwater haplotypes and the closest observed (+), and closest observed or inferred (≠) to occur in marine sitckleback.

Table 4: Summary information from selected surveys of fish mtDNA phylogenetics (adapted from Avise et al., 1987 and Gonzalalez-Villasenor and Powers, 1990).

Species	N	Locales	Geographic scale of study	% genome surveyed	Haplotypes	*p*	References
Stizostedion vitreum	141	9	Great Lakes	2.0	9	0.006	Billington and Hebert (1988)
Fundulus heteroclitus	48	4	east coast cont. USA	2.5	17	0.020	Gonzalalez-Villasenor and Powers (1990)
Oncorhynchus tshawytscha	76	7	west coast Canada and Alaska	2.5	6	0.008	Wilson et al. (1987)
Lepomis gulosus	74	17	southeast cont. USA	3.0	32	0.063	Bermingham and Avise (1987)
L. microlophus	77	17	as above	3.0	7	0.087	Bermingham and Avise (1987)
Amia calva	78	20	as above	3.0	13	0.010	Bermingham and Avise (1987)
Opsanus tau	43	9	east coast Massachusetts to Georgia	1.8	8	0.004	Avise et al. (1987b)
G. aculeatus	30	4	QCI	2.0	7	0.007	Gach and Reimchen (1989)
G. aculeatus†	144	12	QCI	1.4	11	0.035	this study

N, number of individuals surveyed; Locales, number of locations surveyed; Haplotypes, number of mtDNA haplotypes identified; *p*, proportion of nucleotides differing between the most divergent mtDNA types.
† This comparison was restricted to 12 of 13 sites surveyed as one site was very remote from the others, and consisted of only 3 individuals; it's inclusion would have given a misleading impression of the geographical extent of the survey.

Table 5: Estimates of sequence divergence (δ) between selected populations and provisional estimates of times of separation.

Populations			Corrected estimates	
	t (years X10^6) of separation		t (years X10^6) of separation	
Argonaut and Pacific marine populations (based on observed haplotypes)	0.0258	1.29	0.0249	1.24
Argonaut and Pacific marine populations (based on observed or inferred haplotypes)	0.0267	1.34	0.0250	1.25
Atlantic and Pacific marine populations (based on observed haplotypes)	0.0128	0.64	0.0190	0.60
Atlantic and Pacific marine populations (based on observed or inferred haplotypes)	0.0127	0.64	0.0111	0.56

That component of divergence owing to polymorphism present in the ancestral population (δ_A) was corrected for assuming $\delta_A = 0.5(\delta_x + \delta_y)$, where δ x and δ y are mean pair wise mtDNA divergence values between individuals in populations x and y, respectively, following Nei and Li (1979) and Wilson et al. (1985). The Argonaut population was a hypothetical group consisting of all individuals from the four Argonaut localities less those with presumed secondarily introduced pacific lineage haplotypes.

Table A1: Composition of solutions used in preparation and analysis of mtDNA.

Solution	[Conc]	Compound	Reference
Homogenizing medium	0.21M	Mannitol	Lansman et al. (1981)
	0.07M	Sucrose	
	0.05M	Tris-HCL pH 7.5	
	10.0mM	EDTA-NaOH ph 7.5	
	3.0mM	$CaCl_2$	
TE	10.0mM	Tris-HCl pH 7.6	Maniatis et al. (1982)
	1.0mM	EDTA, pH 7.6	
SOC	45.0mM	$MnCl_2.2H_2O$	Glover et. al. (1985)
	100.0mM	RbCl	
	15% (w/v)	Glycerol	
	10.0mM	$CaCl_2.2H_2O$	
	30.0mM	Potassium acetate	
(filter sterilized through 0.22 u membrane, pH 5.8)			
1 X Ligation buffer	50.0mM	Tris-HCl pH 7.6	Manufacturer of ligation kit, Bethesda Research Laboratories (BRL)
	10.0mM	$MgCl_2$	
	1.0mM	ATP	
	1.0mM	dithiothreitol	
	5% (w/v)	polyethylene glycol-8000	
(filter sterilized through 0.22 u membrane, pH 5.8)			
GET	50.0mM	Glucose	Maniatis et al. (1982)
	10.0mM	EDTA	
	25.0mM	Tris-HCl pH 7.6	
1 X TBE	89.0mM	Tris-borate	Maniatis et al. (1982)
	89.0mM	Boric acid	
	2.0mM	EDTA	
1 X SSC	0.15M	NaCl	Manufacturer of Genescreen (Dupont)
	0.015M	Na Citrate	
Hybridization solution	0.5M	EDTA	Church & Gilbert (1984)
	0.2M	NaOH	
	0.26M	Sodium Dihydrogen Orthophosphate	
	7%	SDS	

L broth		Bacto-Tryptone (10 grams/l) Bacto-Yeast extract (5 grams/l)	Maniatis et al. (1982)
	128.2mM	NaCl pH 7.5	

Abbreviations: EDTA = ethylenediaminetetraacetic acid; SDS = sodium dodecyl sulphate; Tris = Tris (hydroxymethyl) aminomethane.

Table A2: Fragment lengths from digests of threespine stickleback mtDNA with ten restriction endonucleases.

Enzyme	Fragment Pattern	Fragment sizes (kilobases)
*Bgl*I	a	7.23, 5.71, 3.84
	b	7.60, 5.71, 3.53
	c	7.23, 3.84, 3.75, 1.96
	d	7.23, 5.71, 2.60, 1.24
	e	7.23, 5.71, 3.84, 3.75, 2.60, 1.96, 1.24
*Eco*RI	a	8.73, 7.94
*Hinc*II	a	3.70, 2.98, 2.52, 2.12, 1.60, 1.22, 0.95
	b	3.70, 3.45, 2.98, 2.12, 1.60, 1.22
	c	3.70, 2.98, 2.28, 2.12, 1.60, 1.22, 0.95
	d	3.70, 2.98, 2.32, 2.12, 1.60, 1.22, 0.95
	e	4.85, 2.98, 2.52, 2.12, 1.60, 0.95
	f	3.70, 2.98, 2.28, 2.12, 1.60, 0.95, 0.85
	g	4.12, 3.70, 3.34, 3.17, 0.95
*Hind*III	a	8.54, 3.27, 2.60, 0.90, 0.78, 0.56
	b	8.54, 3.50, 3.27, 0.78, 0.56
*Hin*fI	a	2.30, 1.99, 1.21, 0.79, 0.72, 0.59
	b	2.05, 1.99, 1.21, 0.79, 0.72, 0.59
	c	2.30, 1.72, 1.21, 0.79, 0.72, 0.59
	d	3.50, 1.99, 0.79, 0.72, 0.59
	e	2.30, 1.30, 1.21, 0.79, 0.75, 0.72, 0.59
	f	2.30, 1.99, 1.21, 0.90, 0.72, 0.59
	g	1.99, 1.95, 1.21, 0.90, 0.86, 0.72, 0.59
*Pst*I	a	9.35, 5.72, 1.58
	b	9.35, 4.14, 1.58, 1.58
	c	9.35, 5.72, 4.14, 1.58, 1.58
*Pvu*II	a	8.70, 7.01, 1.14
	b	8.70, 7.01, 1.12
	c	9.83, 7.01
*Sal*I	a	8.1, 8.0
	b	16.1
	c	8.1, 4.64, 3.38
*Sst*I	a	10.0, 3.56, 2.75
	b	10.0, 6.31
*Sst*II	a	14.7, 1.8

Table A3: Composite restriction fragment patterns for all haplotypes surveyed.

Composite pattern	Site based haplotypes	Samples
aaaaaaaaaa	A	002-006, 008, 009, 012, 354, 374 435-446, 601, 603, 651, 652, 658 659, 663, 600, 701, 706, 711, 830 835, 836, 842, 844, 846, 852, 853 856, 858, 860, 863, 865, 880, 893
a?aaaaaaa?	A	310
aaa?aaaaa?	A	304
aaaaaaaaa?	A	302, 307, 309, 316, 320, 607
aaaacaaaaa	A	855, 857
aaaadaaaaa	A	366
aaaaeaaaaa	A	895
aaaafaaaaa	A	653, 656, 660
aabaaaaaaa	B	879, 883
aababaaaaa	B	710
aacaaaaaaa	C	600, 602, 604-606, 654, 655, 661 662, 777, 827, 851, 862, 876, 877 878, 885, 891, 892, 894
daaaaaaaa?	D	322
aaeaaaaaaa	E	657
aafaaaaaaa	F	776, 778, 779, 781, 782, 784-786, 798, 799
eaaaabaaaa	G	306
aabaaaacaa	H	001, 007, 010, 011, 363, 364, 368-372, 702-704, 709

aababaacaa	H	708
aab?aaacaa	H	712
aabaaaaca?	H	362
aabaaaccaa	I	705
aadbaaccaa	J	500-502
bagagbbbaa	K	882
bagagbbbba	L	751, 753-755, 757-761, 826, 829, 834, 837, 838, 840, 843, 845, 854 868, 859, 861, 864, 884, 890
bagagbbbb?	L	762

Lower case letter designations represent restriction fragment length patterns produced by the enzymes *Bgl*I, *Eco*RI, *Hinc*II, *Hin*dIII, *Hin*fI, *Pst*I, *Pvu*II, *Sal*I, *Sst*I, *Sst*II, respectively, given in table A2. Question marks (?) indicate missing fragment profiles.

Shrimp Lake, 001-012; Boulton Lake, 300-322; Mayer Lake, 350-374; Drizzle Lake, 435-446; Nova Scotia, 500-502; Masset Inlet, 600-607; Sheldon's Lagoon, 650-662; Skidegate Lake, 700-712; Rouge Lake, 750-762; Serendipity Lake, 826-846; Harelda Lake, 852-865; Imber Lake, 876-896.

Figure 1: Location of collection sites of threespine stickleback surveyed from the Queen Charlotte Islands (excluding northeast Graham Island, see figure 2), the adjacent mainland, and the east coast of Canada. Codes for sample localities are as follows: Ns (Nova Scotia); Sh (Shrimp Lake); Sl (Sheldon's Lagoon); Sk (Skidegate Lake); Va (Van Lake). The Cape Ball site is a 16,000 year old vegetative community identified by Warner et al. (1982).

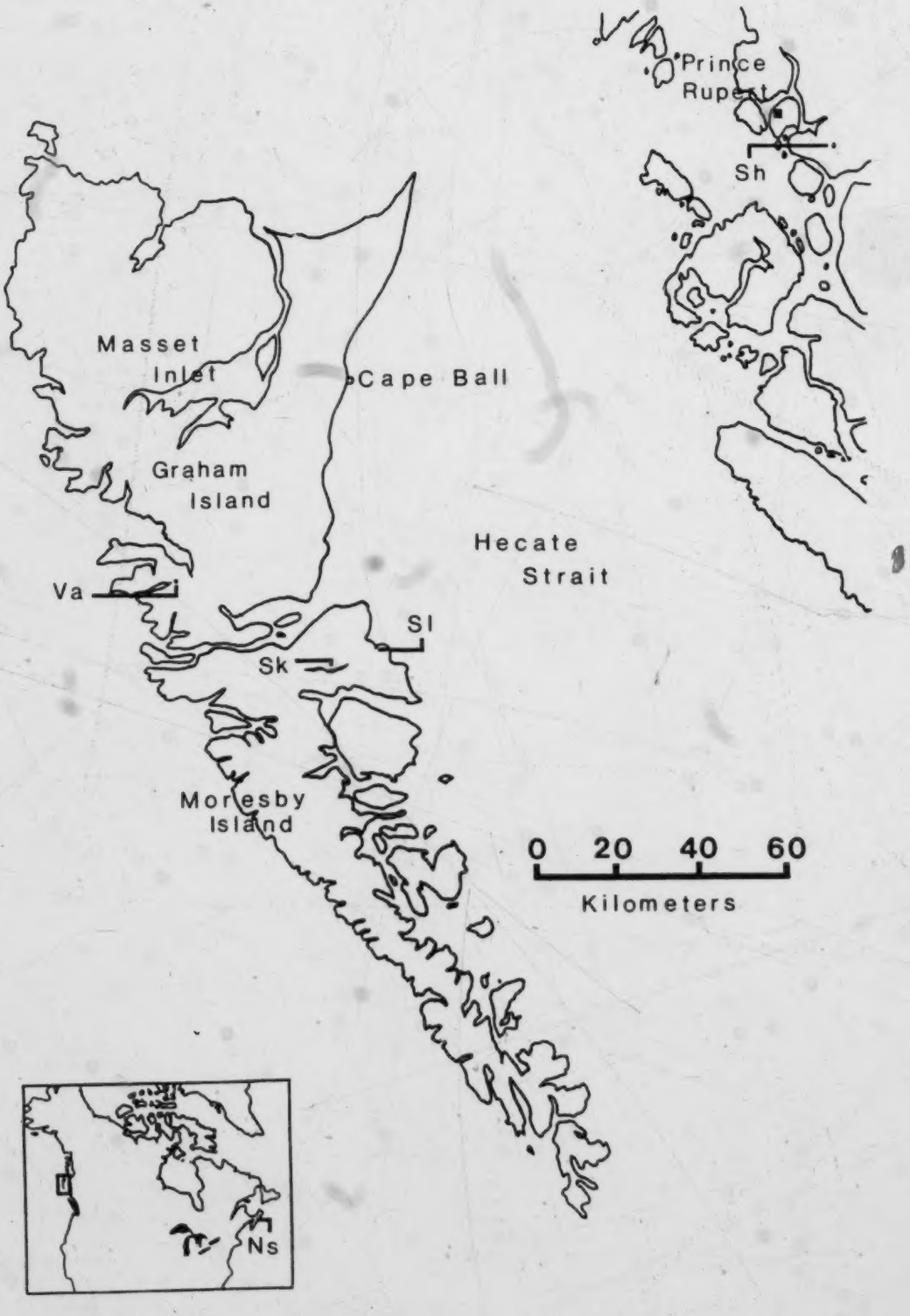

Prince Rupert
Sh
Masset Inlet
Cape Ball
Graham Island
Hecate Strait
Va
Sl
Sk
Moresby Island
0 20 40 60
Kilometers
Ns

Figure 2: Location of collection sites of threespine stickleback surveyed from northeast Graham Island, British Columbia. Codes for sites are as follows: Bo (Boulton Lake); Dl (Drizzle Lake); Ha (Harelda Lake); Im (Imber Lake); Mh (Masset Harbour); My (Mayer Lake); Ro (Rouge Lake); Se (Serendipity Lake). The shaded area represents the Argonaut Plain defined by (Sutherland-Brown, 1968), and the hatched line encompasses the Hiellen River basin. The Cape Ball site is a 16,000 year old vegetative community identified by Warner et al. (1982).

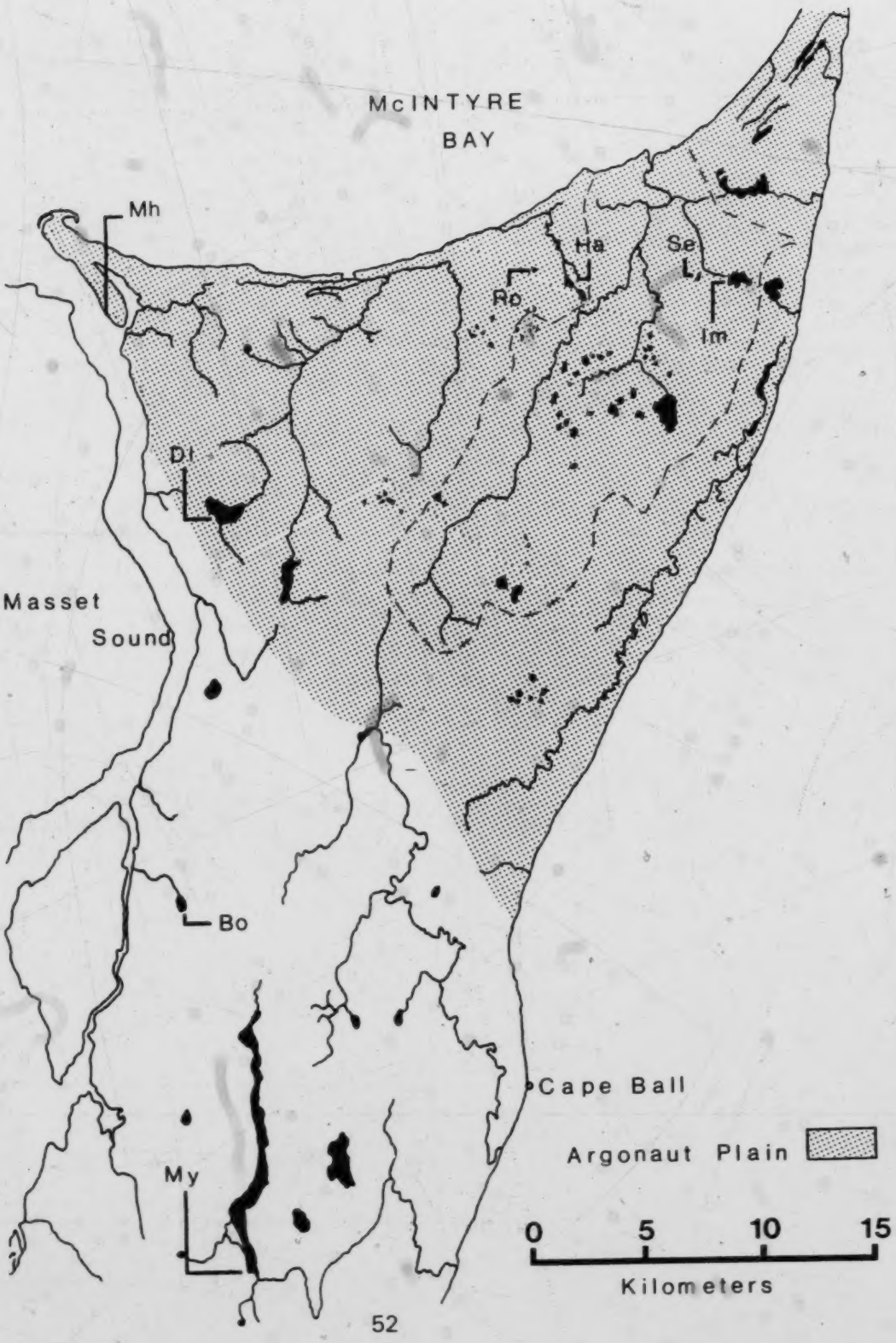

McINTYRE
BAY
Mh
Ha
Se
L.
Ro
Im
Dl
Masset
Sound
Bo
Cape Ball
Argonaut Plain
My
0
5
10
15
Kilometers

Figure 3: Restriction fragment patterns of threespine stickleback mtDNA produced by the hexanucleotide enzymes *Hin*dIII and *Sst*I. Differences between profiles (a) and (b) are due to to single restriction site changes. Size of fragments are given in base pairs.

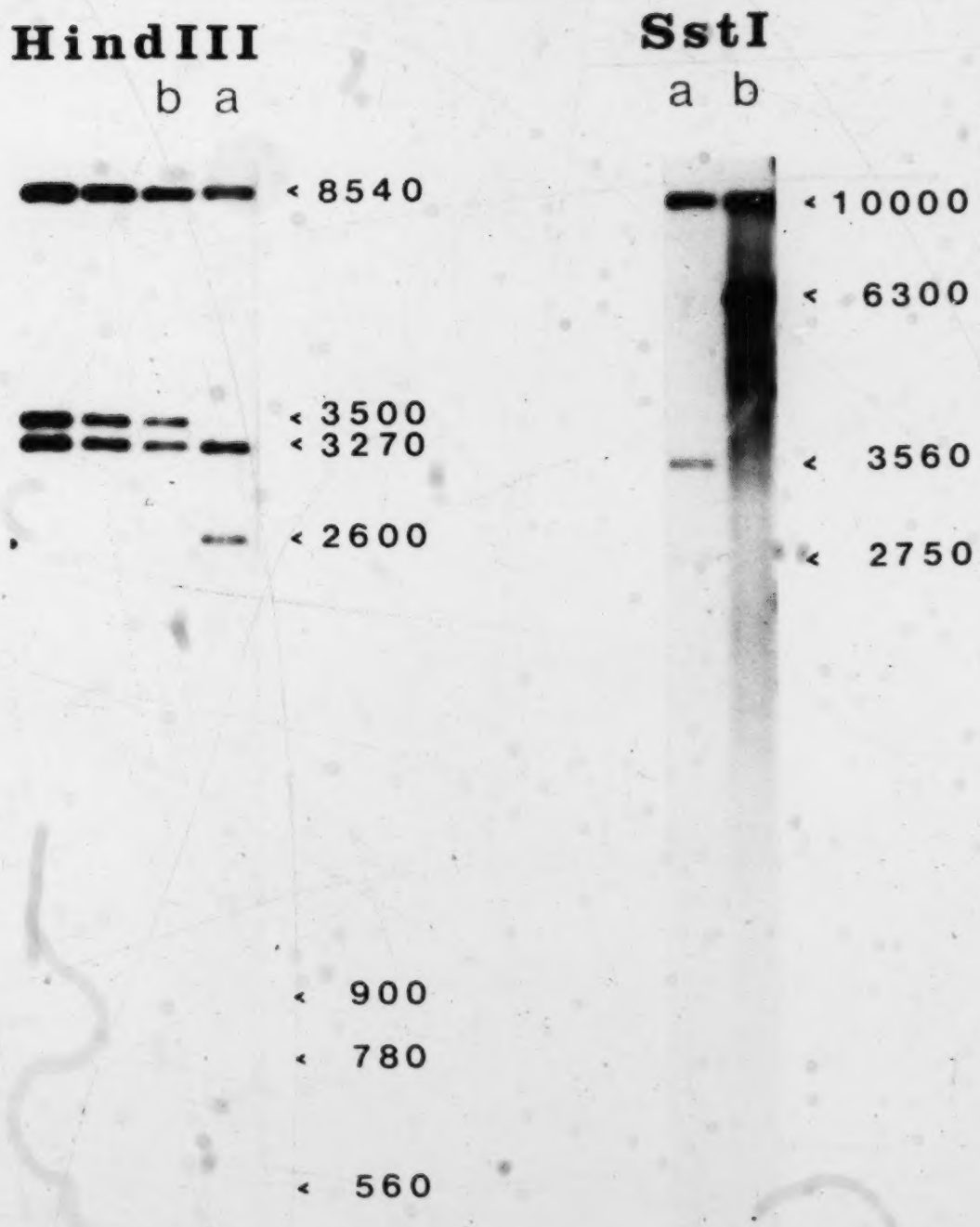

HindIII
b a
8540
3500
3270
2600
900
780
560
SstI
a b
10000
6300
3560
2750

Figure 4: Restriction fragment patterns of threespine stickleback mtDNA produced by the tetranucleotide enzyme *Hin*fI, and the multibase enzyme *Hin*cII. Four of nine fragments were common between patterns *Hin*fI(a) observed in the vast majority of pacific lineage haplotypes, and *Hin*fI(g) identified in all rouge lineage haplotypes. As few as two of ten bands are shared between profiles *Hin*cII(a) of haplotypes (A), (D), and (G) from the pacific lineage, and *Hin*cII(g), of both rouge lineage haplotypes. A minimum of three inferred restriction site changes separate both *Hin*fI and *Hin*cII profiles. Fragment sizes are given in base pairs.

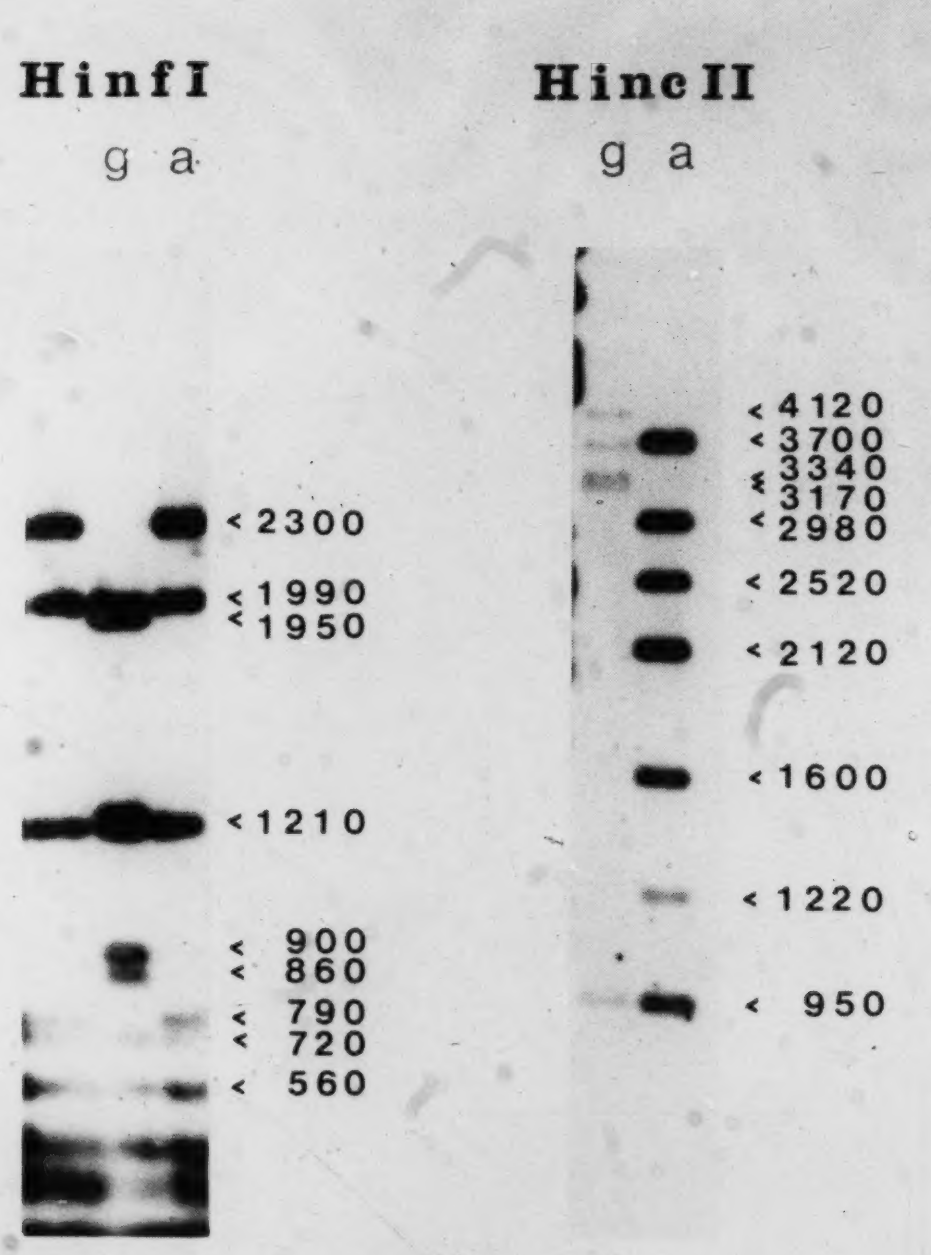

HinfI
g a
< 2300
< 1990
< 1950
< 1210
< 900
< 860
< 790
< 720
< 560
HincII
g a
< 4120
< 3700
< 3340
< 3170
< 2980
< 2520
< 2120
< 1600
< 1220
< 950

Figure 5. Restriction site map of threespine stickleback mtDNA. Positions of monomorphic (outside circle) and polymorphic (inside circle) restriction sites for the enzymes *Bgl*I (B), *Eco*RI (E), *Hinc*II (H), *Hin*dIII (D), *Pst*I (P), *Pvu*II (U), *Sal*I (A) *Sst*I (S), and *Sst*II (T). Numerical subscripts associated with each variable site can be referenced to the table in figure 6 to define composite site maps of all haplotypes observed. The interior circle gives map units, based on an arbitrary scale of 0-100.

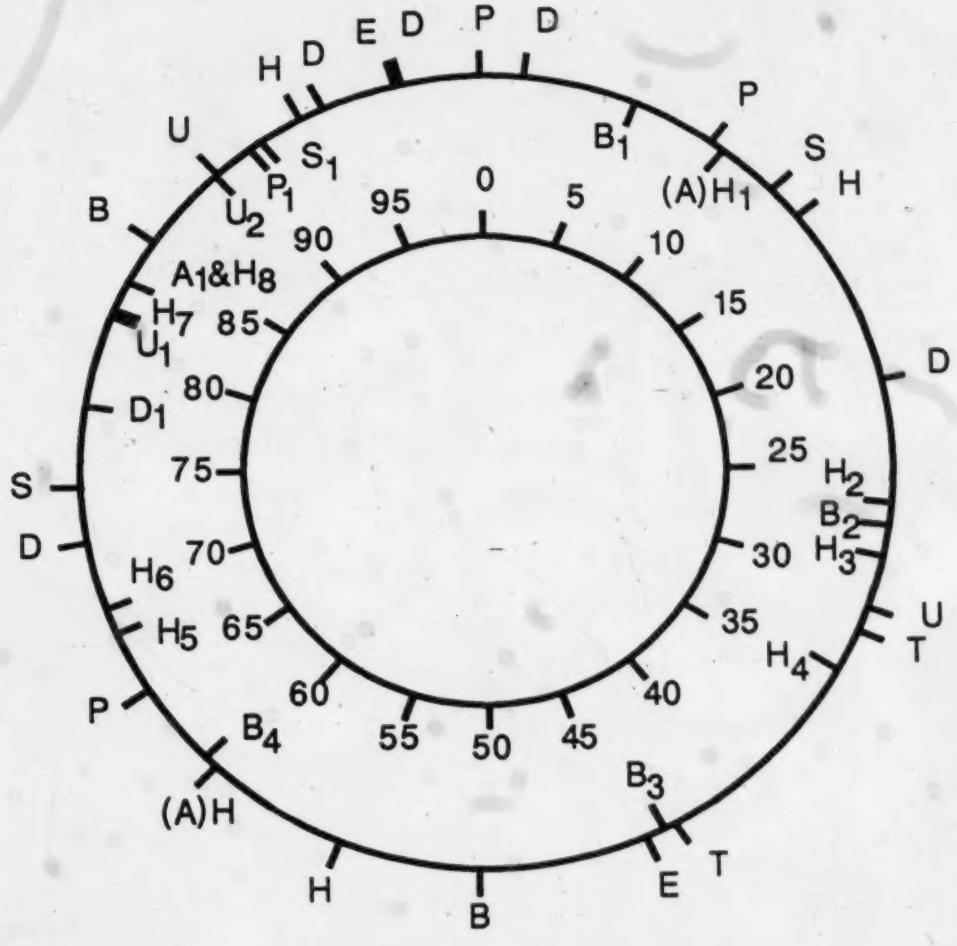
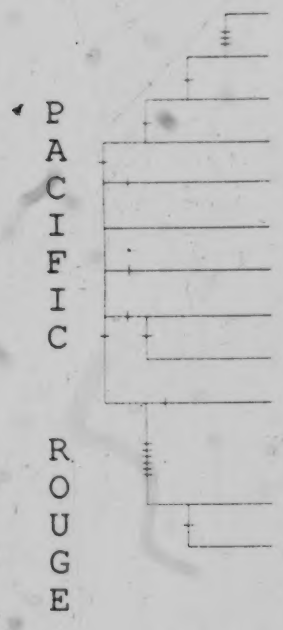


Figure 6: Restriction site matrix of mapped recognition sequences (pluses [+] indicate site presence and minuses [-] site absence). Upper case letters with numerical subscripts represent specific variable restriction sites (see figure 5 for letter designations and site position). The parsimony network connecting haplotypes is constructed from mapped restriction site differences only. Codes for locations are as follows: Bo (Boulton Lake); Dr (Drizzle Lake); Ha (Harelda Lake); Im (Imber Lake); Mh (Masset Harbour); My (Mayor Lake); Ns (Nova Scotia); Ro (Rouge Lake); Se (Serendipity Lake); Sk (Skidegate Lake); Sl (Sheldon's Lagoon); Sr (Shrimp Lake); Va (Van Lake). Frequency values represent the percentage of individuals of a given haplotype of the total number of individuals surveyed (147). *Hin*fI restriction fragment patterns associated with each haplotype are given in the last column, and are represented by lower case letters (see table A2 for corresponding fragment sizes).



Type	Location(s)	Freq. (%)	Restriction Site Pattern																		*HinfI* Restriction Fragment Pattern
			B_1	B_2	B_3	B_4	H_1	H_2	H_3	H_4	H_5	H_6	H_7	H_8	D_1	P_1	U_1	U_2	S_1	A_1	
J	Ns	2	-	+	-	-	+	+	-	+	+	-	+	+	-	-	-	-	+	+	a
I	Sk	1	-	+	-	-	+	+	-	+	-	-	-	+	+	-	-	-	+	+	a
H	My, Sk, Sr	12	-	+	-	-	+	+	-	+	-	-	-	+	+	-	+	-	+	+	a,b
B	Im, Sk	2	-	+	-	-	+	+	-	+	-	-	-	+	+	-	+	-	+	-	a,b
E	Sl	1	-	+	-	-	+	+	-	-	+	-	-	+	+	-	+	-	+	-	a
A	Bo, Dr, Ha, Im, Mh My, Se, Sk, Sl, Sr	43	-	+	-	-	+	+	-	+	+	-	-	+	+	-	+	-	+	-	a,c,d,e,f
D	Bo	1	-	+	-	+	+	+	-	+	+	-	-	+	+	-	+	-	+	-	a
C	Ha, Im, Mh, Se Sl, Va	14	-	+	-	-	+	+	-	+	+	+	-	+	+	-	+	-	+	-	a
F	Va	7	-	+	-	-	+	+	+	+	+	+	-	+	+	-	+	-	+	-	a
G	Bo	1	-	+	+	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	a
K	Im	1	+	-	-	-	-	-	-	+	+	-	-	-	+	+	+	+	+	-	g
L	Ha, Im, Ro, Se	17	+	-	-	-	-	-	-	+	+	-	-	-	+	+	+	+	-	-	g

Figure 7: UPGMA phenogram of sequence divergence (δ) for the 12 threespine stickleback haplotypes identified. Divergence is plotted as 0.5 Xδ, after Nei et al. (1985).

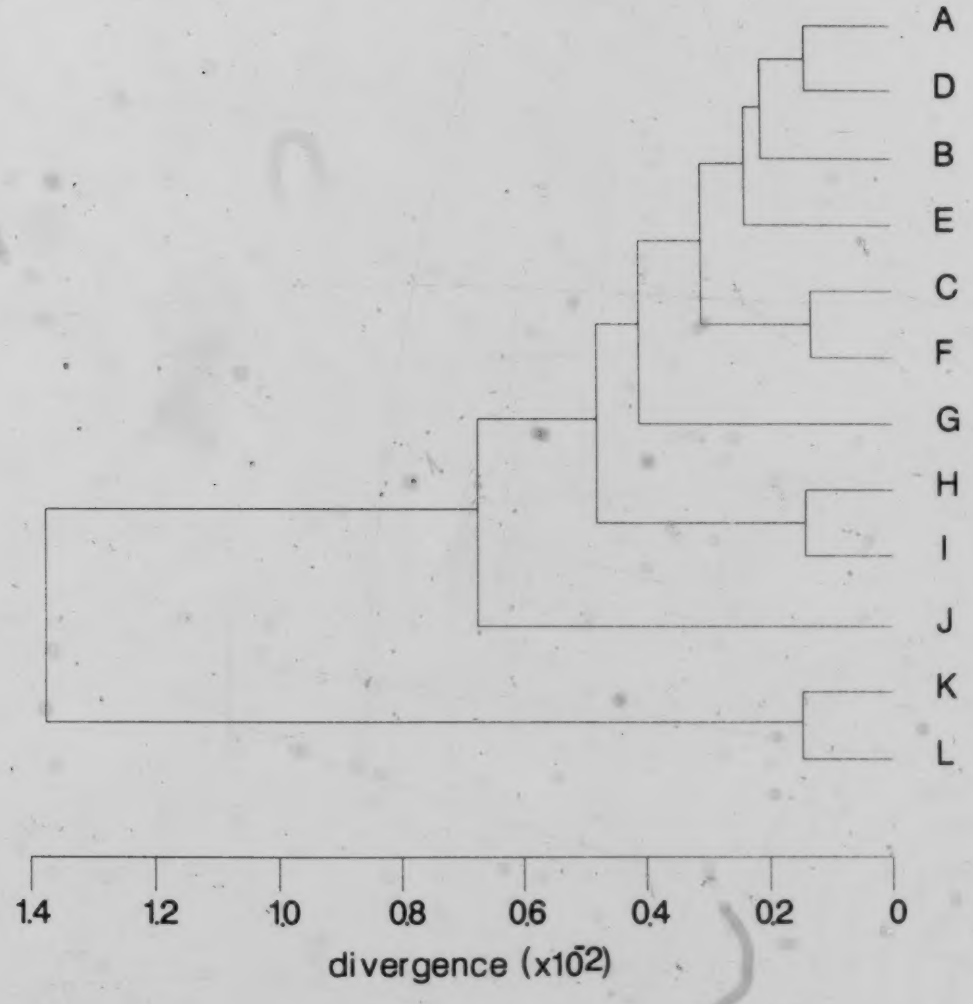

A
D
B
E
C
F
G
H
I
J
K
L
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
divergence (x10-2)

Figure 8: Alternative networks connecting the 12 mtDNA haplotypes identified. In the most parsimonious relationship, type (J) from the Atlantic Ocean connects with type (I), found in a single freshwater locality from Graham Island. Although requiring an additional site change, a relationship with (J) connecting directly with (A) (indicated by the hatched line) seems more plausible, as (A) was found in marine stickleback. Furthermore, based on the following criteria (see Avise et al 1987a), (A) seems the most likely candidate for the ancestral type of the Pacific assemblage: 1) it was the most widely distributed of the those identified, and 2) it forms the hub of the network connecting directly with most haplotypes.

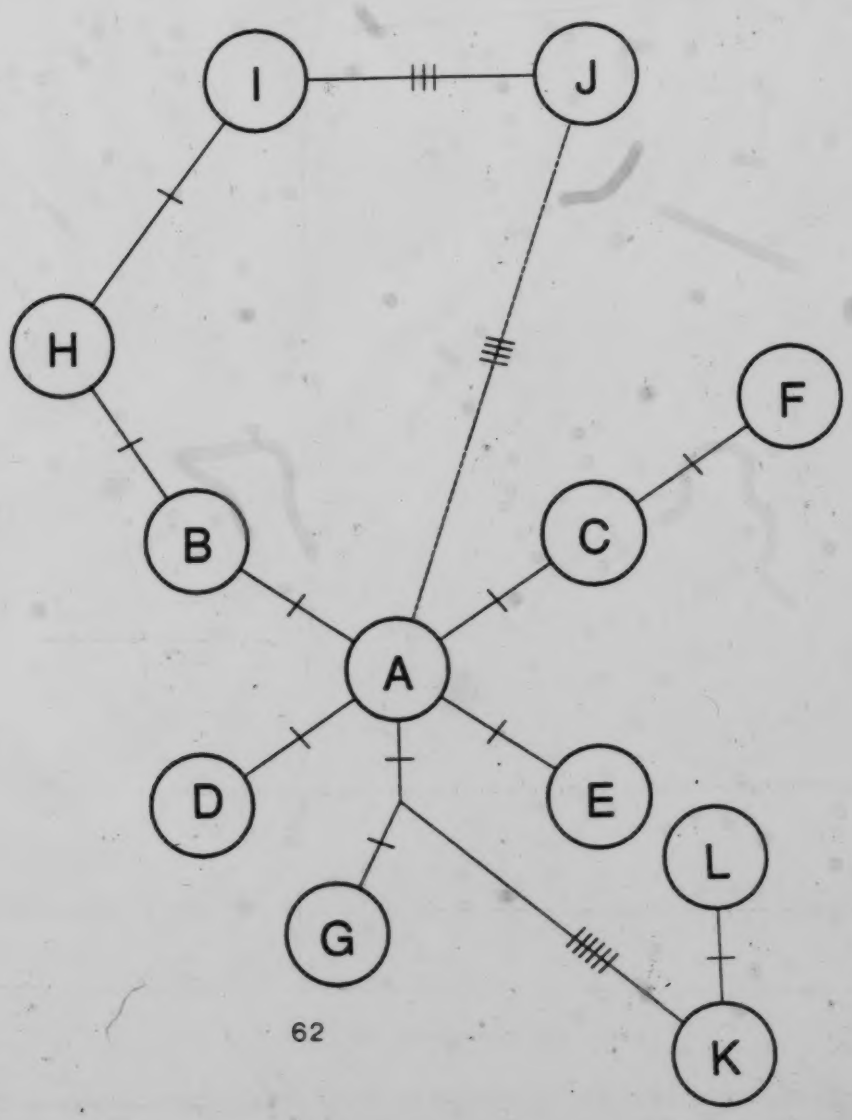
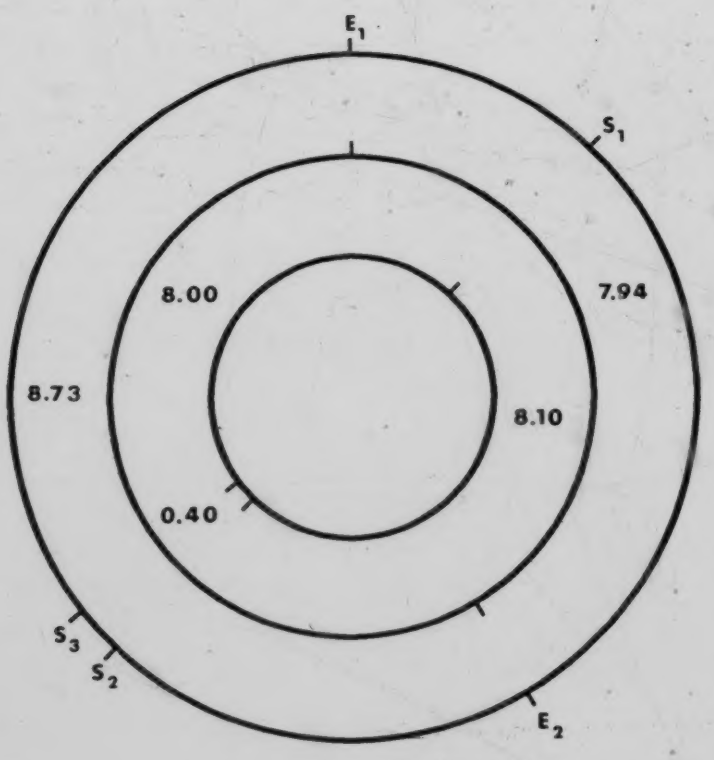
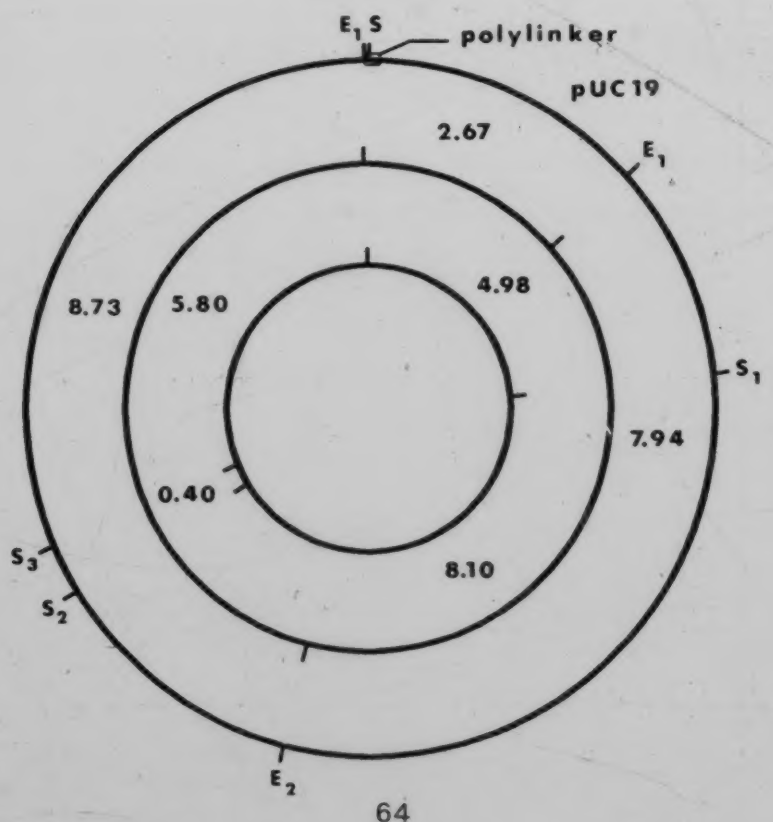


Figure A1: Restriction site map of mtDNA extracted from threespine stickleback eggs and whole tissue for the enzymes *Eco*RI and *Sal*I (a). Restriction site map of pGAMT1 for the enzymes *Eco*RI and *Sal*I (b). The orientation of the three *Eco*RI fragments of clone pGAMT1, where the original arrangement of the two mtDNA fragments is conserved and the plasmid inserts into site E_1, with the polylinker nearest site S_3, is the only combination of the eight possible compatible with the sizes of *Sal*I fragments given in figure b.

a


E1
S1
8.00
7.94
8.73
8.10
0.40
S3
S2
E2

b


E1 S
polylinker
pUC 19
2.67
E1
8.73
5.80
4.98
S1
7.94
0.40
8.10
S3
S2
E2

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Appendix

Inserts were analyzed by comparing restriction fragment profiles of cloned DNA and mtDNA extracted from Hasse Lake stickleback eggs or whole fish. Restriction of mtDNA extracted from stickleback tissue with *Eco*RI produced two fragments, one 8.73 kb and another 7.93 kb in length. One clone (pGAMT1), upon digestion with *Eco*RI, yielded three fragments: one 2.67 kb (the exact length of plasmid DNA, Yanisch-Perion et al. 1985), and two others identical in size to those observed from whole fish or eggs (further evidence identifying these as mtDNA in origin is presented below). The 7.93 kb fragment was also cloned independent of the 8.73 kb fragment on two occasions, once using mtDNA extracted from fish (clone GAMT7.9A) and again using mtDNA extracted from eggs (clone GAMT7.9B). The 8.73 kb fragment was cloned independently of the 7.93 kb fragment using mtDNA extracted from eggs (clone GAMT8.7).

The ligation of the entire mtDNA genome into the plamid polylinker *Eco*RI site was unexpected for two reasons: 1) large insertions (greater than 10 kb) tend to lower efficiency of transformation (Sambrook et al. 1989), and 2) the presence of an *Eco*RI site within the 16.6 kb insert. The latter could be explained by either 1) incomplete restriction of the population of mtDNA molecules extracted from fish eggs, or 2) ligation of 8.73 kb and 7.93 kb fragments followed by ligation to plasmid DNA.

Since much of the restriction site map of threespine stickleback mtDNA was based on digests, double digests, and partials digests of cloned mtDNA, the orientation of the two *Eco*RI fragments with respect to each other (in the event of scenario two described above accounting for incorporation of the entire 16.6 kb fragment) and with respect to the plasmid was assessed.

From single and double digests of Hasse Lake fish mtDNA, a restriction site map of *Sal*I and *Eco*RI recognition sequences was produced (figure A1a). This was used to

construct models of the eight possible orientations of the three *Eco*RI clone fragments, with *Sal*I markers as expected based on fish mtDNA. Upon digestion with *Sal*I, cloned mtDNA yielded the following sized fragments: 8.10, 5.80, 4.98, and 0.400 kb; only the configuration given in figure A1b would produce this restriction fragment profile. Subsequent comparisons of single digests (for many different enzymes) of clone and fish mtDNA confirmed the orientation given above.

Clone pGAMT1 was used exclusively in this study as probe DNA to identify membrane bound stickleback mtDNA. As total DNA (nuclear and mitochondrial) was extracted and transferred to membranes, it was important to demonstrate with a high degree of certainty that pGAMT1 was indeed mitochondrial and not a 16.6 kb nuclear fragment.

In addition to the size correspondence of *Eco*RI fragments of cloned and egg mtDNA visualized by ethidium bromide staining techniques, several other lines of evidence implicate mtDNA as insert in plasmid pGAMT1. First, the fragments in each of the near 1500 restriction digest profiles, identified by hybridizing labelled probe DNA to membrane bound DNA, always summed to 16.6 kb (+/-0.5kb), the size of three spine stickleback mtDNA estimated by Gach and Reimchen (1989). This strict conservation of size is a characteristic often associated with mtDNA. Second, only in one instance was more than one type of DNA molecule observed in any single individual. Except in cases of unexpectedly low levels of heterozygosity would this be observed for nuclear DNA markers. Third, *Hin*dIII restriction fragment profiles of fish from Drizzle Lake surveyed here and by Gach and Reimchen (1989) were identical, at least over the range of fragment sizes scored in this study; fragments below 500 base pairs identified by Gach and Reimchen were not resolved here.